

No more NPT rows, please

The third review conference of the Non-Proliferation Treaty opens next week at Geneva. Old discontents are bound to surface. Can the participants think forward too?

WITH the passage of time, the Non-Proliferation Treaty (NPT) becomes increasingly important. Of all the extant instruments of arms control, it is the only one to include a substantial number (nearly 130) of signatories whose signatures imply self-restraint (as distinct from formal acknowledgement that others are restrained), it is the chief means by which it is possible to distinguish would-be bomb-makers from the rest and it has the virtue of providing a public discussion of nuclear arms control at regular intervals of five years. The third conference in this series, which begins next Tuesday (27 August), is certain to be as wide-ranging as its predecessors. The hope is that it will not be as rowdy as that in 1980, when the conference failed to draft an agreed statement.

The participants seem to have learned a great deal from that fiasco. On this occasion, people seem to have been diligent in their preparation for the review conference. In the hope of providing some kind of structure for the inevitable debate between the non-nuclear powers on the adequacy of the steps taken or attempted towards nuclear disarmament, there is to be a separate committee on the topic. Similarly, the safeguards system and the peaceful use of nuclear power are to be dealt with in an organized way. None of this will prevent complaints or recriminations; but since there is no reason to think that any of the participants wants the NPT to collapse, there are grounds for hoping that vituperation stops short of vilification.

The NPT is often described as a bargain between the nuclear and non-nuclear powers, but it is more than that. The three overt nuclear powers (France and China do not belong) have agreed to search for agreements on the reduction of their nuclear weapons, while the signatory non-nuclear powers have accepted international safeguards as an earnest of their promise not to make them. The treaty also lays down that the nuclear powers will provide technical assistance with the development of civil nuclear power in non-nuclear countries, or at least offer access to the International Atomic Energy Agency (IAEA). Parties of both kinds promise self-restraint, which is where the bargain rests. But both kinds of parties also have what must be supposed to be an identical interest that the number of nuclear powers should not increase. Only that common interest will enable them to hang together in a year when the two principal nuclear powers have nothing substantial to report except that they have reconvened, perhaps in a more hopeful environment, a series of negotiations broken off halfway through the interval since the 1980 conference.

Even so, the non-nuclear powers had better bite their tongues when complaining at Geneva that the nuclear powers should have done more. There are some minuses but also, surprisingly, some pluses. The minuses are that the full extent of the Soviet deployment of SS20 missiles has become apparent, that the countervailing modernization of NATO forces in Western

Europe has begun and that the United States has created at least confusion, perhaps justifiable alarm. Certainly there will be voices raised at Geneva during the next four weeks about the folly of this proposal, of which the best that can be said is that it cannot be as effective as it claims; and many of them will be Soviet voices. The pluses are more easily overlooked. Many governments will have forgotten that in 1980, the Carter administration in the United States had signed the second part of the SALT agreement on strategic weapons, but had not taken it to Congress for ratification, perhaps because it dared not risk defeat, perhaps because of the reason given that Soviet dealings with Afghanistan were an offence. Relations between the two superpowers may not be all they should be, but they have improved a great deal since 1980.

On the issue of civil nuclear power in non-nuclear weapons states, the climate has also improved or has become more realistic. The non-nuclear powers have put behind them the dis-appointments (for some) of the UN conference on science and technology for development, have put such resources as they have been able to spare for energy production into energy conservation (which always had first claim), but have begun to find opportunities for the economic use of nuclear power; the signs are that the international supply system, stripped of the over-optimism of earlier decades, is working well. Even the safeguards system, much resented by non-signatories such as India, is working more smoothly and less obtrusively — but there is still a need that the details gathered by IAEA inspectors should be made public.

So why are people still worried about the proliferation of nuclear weapons? NPT is an important part of the system for preventing proliferation, but is only a part of it. And during the past five years, there has been literally no progress in persuading those outside NPT to join with the other signatories. Israel remains putatively a nuclear power. Pakistan and India plainly seek the same model. Argentina and Brazil have not ratified the treaty. Nobody can guess what Libya let alone South Africa is about. China and France are conspicuous by standing aside. That is why one of the chief objectives for the next five years should be to persuade backsliders like these to join NPT. The task is political, but the benefits would be great. □

What hope for AIDS?

The temptation to legislate to protect society from the spread of AIDS should be resisted.

HARD cases make bad law, and public anxiety is a poor basis for social legislation. That should be the starting point for any consideration of how government should respond to the demands popping up all over the world that something should be done to protect people from AIDS, otherwise acquired immune deficiency syndrome, now known to be caused by the virus HTLV-III, or LAV in France. That there should be a certain air of panic is understandable. AIDS is a dreadful and also, still, a mysterious disease. Only some 10 per cent of those infected with the virus develop the symptoms that lead almost always to death from adventitious infection, for reasons not yet understood. Little is known, as yet, about the efficacy with which those

Biological manuscripts

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carrying antibodies against the virus can infect others. Moreover, the death tolls mount. More than 5,000 people have already died in the United States. In Britain (where mortality exceeds 100), other European countries and Australia, while the numbers of those dead are still small, there is the awkward prospect of a rapid increase as the larger numbers of patients with overt disease eventually succumb. It is bound to carry weight with people everywhere that in New York, among males in their thirties, AIDS is reported to have taken precedence over homicide as the chief cause of death. Where will it end, people are asking — and in the meantime they demand protection for themselves from the disease.

That is why it is important that everybody should understand how little can, at this stage, be offered. AIDS is a tragic disease, but a collective tragedy. Its emergence is a piece of evolutionary bad luck for all of us. Although still largely confined to male homosexuals, AIDS is certain in due course to spread more widely (see *Nature* 312, 97; 1984). This appears to have been the case in Zaire since 1977, for retrospectively it has been shown that the virus has been widespread and heterosexually transmitted for the best part of a decade (Piot *et al. Lancet* ii, 65; 1984). Already in the United States, there are reports of some scores of infant deaths, presumably as a consequence of congenital infection. There is nothing to suggest that AIDS will not become just another venereal disease, but one not tractable to the antibiotics familiarly now in use. Fortunately there is as yet no evidence that the virus is transmitted by means other than sexual intercourse of one kind or another.

What, in these circumstances, should public policy be? Plainly there is an urgent need that strictly adventitious infections should be avoided. The cases of haemophiliacs infected through contaminated blood are doubly tragic; people disabled by an inherited genetic deficiency are cruelly dealt with if society cannot provide them with a safe palliative. There is the strongest reason why haemophiliacs and others in need of blood transfusions should be protected as far as possible by the use of the screening assays so far developed; there is even a case that those discovered to have HTLV-III antibodies in their blood should be identified, warned of their condition and black-listed as blood donors. So much is feasible and urgently necessary. Governments should not stint themselves. In Britain, the government should not expect hard-pressed health authorities to provide the extra funds they will need out of budgets already trimmed to the bone.

How much further is it possible, or wise, to go? There is a natural temptation to make the disease notifiable in the technical sense, which would give health authorities the power almost automatically to confine patients in isolation; quite apart from the lack of isolation facilities that would be required to keep patients out of contact with other people for months on end, the result would almost certainly be that those most at risk would be deterred from seeking medical help for suspected infection. Sensibly, most governments and the British in particular have resisted this course of action. Others, however, would go even further. Dr John Griffin, director of the Association of British Pharmaceutical Industry is reported by *The Times* to have written urging that there should be compulsory screening of male and female prostitutes, groups almost certain to be or to become foci for the spread of infection. The obvious difficulty is that there can be no effective way of enforcing such legislation. How is prostitution to be defined? What assurance can there be that, given a workable definition, the result will not be that prostitutes are even more firmly put out of touch with physicians? These are problems that those seeking to control the venereal diseases that already exist have grappled with in vain for years. Even though AIDS is a sombre shadow over public health, there can be no reason why these diseases should be more easily conquered now than in the past. In any case, not nearly enough is known about the transmission of AIDS for it to be more than a presumption that prostitution is such a crucial link in the process. Legislation would probably do more harm than good. Public education is the better course.

The message that needs to be made public is unusually depressing. Prophylactic vaccines are a long way off. The prospects for curing people who have developed the disease are even more distant. Palliation is uncertain, as things are. How is it possible to inform the general public about an infectious disease for which so little can be done? There are two cheerful things to say. First, it is a mere four years since AIDS was recognized as such, since when the infecting organism has been identified by heroic efforts in the United States and France; at least diagnostic kits for AIDS antibodies now exist, and will be improved. And, by good fortune, the lymphocytes which are the targets of the virus are also at the centre of one of the most rapidly moving fields of research, from which it is not unreasonable to hope that ways of manipulating the process of infection will spring. But, second, there has also grown up, in this short time, experience of how patients can be helped psychologically to come to terms with their condition, and with the importance of behaving so as to make an individual contribution to the process of trying to slow down the spread of the disease. The public clinics in the United States have mostly shown a remarkable blend of compassion and hard-headedness in their care for patients. The pity is that so many of the communities in which they work are over-zealously concerned to seek personal safety in illiberal legislation. □

Academic independence

The British government's inquiry into the University Grants Committee could be useful.

THE British government's plan (see p.669) to review the constitutional status of its University Grants Committee (UGC) should not provoke the knee-jerk reaction that comes first to mind. Since 1919 the University Grants Committee has been a buffer between the government and the universities it supports. For most of the time, the committee has been applauded as a unique (and even uniquely British) device for spending public money without running the seemingly inevitable risk of government interference. (The British Broadcasting Corporation enjoyed a similar reputation until its governors caved in three weeks ago to an ill-judged request from the Home Secretary not to broadcast a film on Northern Ireland, and until it leaked out that BBC appointments are regularly vetted by the government's intelligence services.) Certainly, the existence of UGC has ensured that the affairs of individual universities are not open to scrutiny by, for example, the House of Commons, which has suited both the university system and successive governments. Broadly speaking, the committee's recommendations about the development of the individual universities as well as the system as a whole have also been fair and constructive. The fly in the ointment is that UGC's seeming independence has always been more apparent than real. During the six years of the present government, ministers have chosen to remind the universities of the old doctrine about the right of the one who pays the piper to call the tune. The committee's independence has been undermined. There is at least a chance that the inquiry now to be mounted will restore some of that independence.

Constitutionally, UGC is not an executive body but yet another advisory committee. Each year it makes recommendations to the Department of Education and Science about the distribution of the funds available, whereupon the government writes the appropriate cheques. There has never been an occasion when the government has rejected UGC's advice, but constitutionally there is no reason why that should not happen. Over the past decade (since before the present government took office), UGC has lost the essential benefits of the old quinquennial system under which the scale of spending was fixed in outline five years in advance; now there are three-year rolling forecasts, which may be changed from year to year. That was the rubric under which the government arbitrarily announced, at the end of 1980, that the total budget of the universities would have to shrink by 8.5 per cent over three years; the UGC of the time incurred the wrath of those universities whose budgets

were disproportionately made to shrink, which was a convenience for the government which had already decreed what fees should be paid by students from overseas. After all this buffeting, UGC lies in a constitutional limbo made more ambiguous because it has no staff of its own, and no freedom to spend money on the techniques of running an office.

The biggest challenge to UGC's independence lies only a little way ahead. The committee has made no secret of the opinion that the time will soon arrive when it will be prudent for at least one of the universities in the British system to be closed. The overall budget is still shrinking by about 1.5 per cent a year in real terms, while savings that may arise from a more selective distribution of funds will not come soon enough to ease the pressure. Traditionally, in such a circumstance, it would be for the committee to say which institution should be shut, but it has plainly said that it will leave that invidious decision to Sir Keith Joseph, Secretary of State for Education and Science. The reasons are plain. Given that the British government is the ultimate paymaster, there is no chance that a decision to close a particular university could fail to become a political issue. There will be rows in the House of Commons, while the government itself will be tempted to make electoral calculations when deciding which parliamentary constituencies to offend. When that time comes, probably before the new inquiry is complete, it will be generally apparent that UGC is not in charge of the British university system nor even a universal buffer between it and the government. At that stage, the government will also be in a mood to regret that it has so emasculated UGC's independence.

This is an opportunity that British universities should exploit. The government's interest in the past few years has extended beyond budget-trimming to schemes for encouraging more concentration on science and technology and attempts to create a closer interaction between universities and industry, partly for its own sake but also in the hope of saving money. UGC has responded within the limits of its means as positively as could be expected, but the government has found that the provision of more places in science and technology cannot be engineered out of the blue, at no extra cost. Sir Keith Joseph has insisted on his government's right to shape the university system in ways like this, and within the bounds of what is possible, the claim cannot be denied. But the government cannot ask for what is not possible, nor can it, without the advice of people who know the university system intimately, know what is possible and what is not. From these considerations, the Butterworth committee's recipe for change should follow immediately.

First, UGC should be constituted as an independent public corporation, with the resources to manage its own affairs and the freedom to employ its own staff (mostly, at present, on loan from the department, but with increasing help from the universities). Second, it should be made financially autonomous, with the power to write its own cheques in favour of the institutions in its care — and the equally important right to carry over money from one year to the next. (Ideally, UGC should be able to accumulate the kinds of trust funds on behalf of British universities in general which do not at present exist.) But how to take account of government policy on universities? Simply by requiring that the membership of UGC should include the ministers responsible, the Secretary of State for Education and Science or his deputy, perhaps other heavyweights as well. Conventionally, of course, ministers do not belong to committees of which they are not the chairmen, but that would in this case be inappropriate. The record of the past few years shows that ministers have much to learn about higher education as a whole; membership of UGC should be quickly educative. But UGC itself is a sleepy and over-cosy committee, inclined to regard the British government's decisions about budgets and the like as slings and arrows of outrageous fortune. Its meetings would be more lively and more pointed if the perpetrators of these insults, real or imagined, were to be found around the same table once a month. And by what other means can there be a genuine partnership between the British government and the academic community on the management of the university system? □

Justice by bankruptcy

The Manville solution for the settlement of claims by asbestos victims could become a precedent.

THE Manville Corporation of the United States has had an eventful, no doubt salutary and (for the rest of us) admonitory year. Historically, the corporation has been a processor of asbestos and a manufacturer of asbestos products. In the past decade it has found not merely that its business has declined as the use of asbestos in the construction industry and elsewhere has declined, but that its existence as a commercial corporation had been put in jeopardy by the volume of the claims by private individuals and their families alleging that their health had been damaged by asbestos fibres, which are known to be capable of causing a characteristic form of lung cancer. Three years ago, no fewer than 60,000 lawsuits had been laid against the company, which took the unusual but proper step of seeking protection under the US bankruptcy laws. The company now guesses that the total amount of the outstanding claims amounts to more than \$2,500 million, more than it could hope to raise by liquidating its assets. So, if the suits were to have been taken seriously, the company was technically in the position of not being able to meet its creditors' claims out of its own resources, which is the same as to say that it was technically bankrupt. But to have sold up would have been of little help to the claimants, which is why the company sought protection under Chapter XI of the bankruptcy act, which allows a company to continue to run its business under court supervision, in the hope of generating the funds with which to pay off its debts. (Companies and also creditors elsewhere may envy this enlightened legislation.)

The outcome of Manville's flight to the shelter of the bankruptcy laws is nevertheless surprising and, in some ways, ironic. Part of the difficulty in dealing with the lawsuits has from the outset been that nobody could predict how many lawsuits were yet to be laid by people in whom overt signs of damage were not yet apparent. But how can the accountants take account of imponderables like this when trying to decide whether a company is technically bankrupt? The courts have fortunately devised a way, one that is almost certain to be followed in other such circumstances. The principle is that legitimate claimants against the company shall be compensated not by the outcome of the particular lawsuit they may have laid, but by means of payments from a trust fund Manville will be required to set up. First, there is a substantial sum due from Manville's insurers (big enough to have caused trouble on the Lloyds insurance market in London). Manville itself is required to bless the fund with \$200 million in cash and if necessary to hand over 20 per cent of its annual profits. The trust fund is also to own a substantial part of the company and there is a complicated arrangement about convertible preference shares that will, according to the volume of legitimate claims eventually made, allow the fund to finish up owning as much as 80 per cent of the company. The issue will be decided next month.

That, of course, is the irony. Manville and its existing shareholders are being required to do the decent thing in financial terms by its past and future victims, but in a way that gives them an interest in the success of the business. The potential advantages are, however, important. Settlements should be quicker. Less of the funds available for compensation will stick to the lawyers' hands by way of contingency fees. And it will be easier for people who think they may have been damaged to tell where they stand. If the small flurry of lawsuits being brought against tobacco companies on the grounds that cigarettes cause lung cancer should stick, other companies than Manville will be rushing off to the bankruptcy courts. The obvious difficulty is that the feasibility of a tobacco company continuing to trade would depend crucially on an actuarial calculation of whether the cost of compensating victims would suck up all the profits from selling cigarettes to those who escape. The moral, for those who must smoke, is to switch to a brand made by a manufacturer whose business is not solely tobacco-based. □

Radiation exposure

British nuclear workforce assessed for risks

RESULTS of a study carried out by the British Medical Research Council (MRC) claim that there is "no significant risk to health" from radiation to people working for the UK Atomic Energy Authority (AEA). The study, published last week in the *British Medical Journal* (291, 435 and 440; 1985), relates mortality to recorded exposure to ionizing radiation for 39,546 employees of AEA between 1946 and 1979. Of the 3,373 deaths that occurred, 937 were from cancers; overall, death rates of the sample population were lower than those in the country as a whole, but consistent with those expected in a normal workforce and probably accounted for by the social class of AEA employees. But one type of cancer, prostate cancer, was unusually prevalent in the sample group.

The UK study was undertaken as a result of a report in 1977 from the United States, which suggested that the risk of developing cancer after exposure to low-dose ionizing radiation is 20 times greater than the recommendations of the International Commission on Radiological Protection (ICRP). That study, which investigated a workforce in the US nuclear industry, has been criticized for its methodology and analysis of results. AEA therefore asked MRC to commission an independent survey of mortality in its own workforce. MRC's epidemiological study was performed by Professor Geoffrey Rose, Dr Valerie Beral and colleagues at the London School of Hygiene and Tropical Medicine. Mortality of all AEA employees since 1946 was examined and the results compared with ICRP's figures.

The authors of the new study say that their data give no unambiguous guide to the feasibility of estimating the radiation risks to people exposed to low doses by extrapolation from the mortality among the survivors of atomic bomb explosions, exposed to acute doses, the basis of the ICRP recommendations. The correlation between radiation exposure and mortality is so uncertain that, at the 95 per cent confidence limits, the data are consistent with a chronic radiation risk 15 times greater than that suggested by ICRP and, at the other extreme, a risk which decreases with increasing dose.

Of the deaths that occurred, leukaemia rates were slightly above the national average, but as the total number of deaths from this cause was small (35 overall, or 4 per cent of all cancer deaths) the excess could be explained by chance fluctuations. Mortality was not, in general, correlated with radiation exposure, the exception being prostate cancer, which caused 38 deaths. This figure is several times greater

than the national average in the small groups of workers who had relatively high exposure to radiation and who had also been monitored for contamination by radionuclides, particularly tritium. No explanation for this result is suggested by the *British Medical Journal* papers, which point out that tritium is not prostate-seeking, but that tritium contamination may be an indicator of some other agent.

MRC is now "actively considering" how to investigate further the results on prostatic cancer. The epidemiological study is being extended for a further five years,

partly to obtain more reliable statistics and partly because, as people in the sample become older, more cancers will emerge which will allow risk estimates to be recalculated with greater precision.

Over the next few years, other UK studies on the effects of radiation will attain the stage that this MRC/AEA survey has reached. Studies in progress are on atomic weapons research staff, staff of British Nuclear Fuels Ltd (preliminary results already published), the National Radiological Protection Board's radiation register for the 1970s and the Central Electricity Generating Board's survey on its workforce. Pooling of these results with the existing and future AEA figures from surveys in other countries will enable more confident limits to be set on radiation safety levels and provide a more accurate estimate of the specific risks involved.

Maxine Clarke

Japanese economy

The future is even brighter

Tokyo

JAPAN'S economic prospects will be even brighter in the years ahead. That is the message of the latest white paper from the Economic Planning Agency. And although the document repeats the familiar self-criticism of recent years that Japan lags behind the United States and Europe in fundamental science and the development of original technology, the criticism is only muted.

Indeed, the report predicts an economic future for which most European leaders would sell their souls. The past ten years — in which annual growth has regularly been above 5 per cent, two or three times that recorded in the United Kingdom, and in which Japan has emerged as the world's second greatest economic power — is dismissed as an era of "stagflation". From now on, Japan will enter a new age of inflation-free rapid growth.

Three factors will contribute to this expansion. The first is rapid growth in the telecommunications, electronics and other high-technology industries. In 1984 alone, Japanese corporations invested five thousand million yen (10^{12}) in these areas. Second is the growing service sector and increasing urbanization, with the Japanese population becoming ever more concentrated. And third is the growth of Japan's Pacific neighbours — from California through Australia round to Taiwan, Korea and China — which, it is believed, will make the Pacific basin the centre of world economic activity by the turn of the century, leaving the Atlantic nations to wither in their grey and sunless climes.

Only one small cloud floats in this perfect blue sky. Japan now supports a smaller percentage of old people than European countries. But by the end of the century, with the Japanese now the world's longest lived people, the situation

will have reversed and the nation will be faced by huge pension and medical bills. The report suggests that this may be dealt with by raising from 60 to 65 the age at which citizens can receive government pensions and by restricting the number of doctors. And that in turn has led to stern criticism that economic planning agencies have no business deciding welfare policy.

Alun Anderson

More money for NIH

Washington

In the last days before its summer break, the US Congress defied the administration by passing a bill instructing the National Institutes of Health (NIH) to award no less than 6,200 extramural research grants in the current fiscal year, slightly less than Congress originally intended but 1,200 more than the administration's planned figure of 5,000 grants.

Thwarted by Congress's limitless generosity to NIH, the administration had tried to commit some of the 1985 budget for the future years of multi-year grants, a move that was challenged on legal grounds by the US Comptroller General. The supplemental appropriation now passed by Congress (HR2577) does not give NIH any money above the \$938 million they had already been given for research grants, but by specifying the number of grants, it prevented the administration from pursuing the multi-year funding plan (see *Nature* 313, 377; 1985). The legality of the administration's plan has not been resolved.

Researchers should not expect their money too soon, however. By denying his signature, President Reagan could yet exercise a "pocket veto" and kill the bill, as Congress is not in session to oppose him.

Tim Beardsley

US engineering

Backing for long shots

Washington

RESEARCHERS who fail to win a grant from the National Science Foundation's engineering directorate will not in future be able to blame the foundation's excessive caution or lack of vision. The dynamic new assistant secretary for engineering, Nam Suh, has instituted a new grant for proposals that are rejected by the peer review system but which are so exciting that they perhaps ought to be funded just in case. This year, 12 such "high risk/high return" projects have been supported, with grants worth a total of \$1.2 million.

The new grant is administered by a special 7-member Committee on Innovative and Exceptional Research (to distinguish it, presumably, from the rest of the research supported by the foundation). The committee's chairman, William Butcher, explains that the grant was established to counter a fear that peer reviews of research proposals are inherently biased against "way-out ideas". Besides funding risky proposals, the committee can consider "highly innovative truly exceptional proposals" (it has not yet received any of these) and, for the sake of completeness, proposals that do not fall into any of the other categories (so far, they all do).

This year the committee was unable to use all the \$2 million at its disposal, perhaps because the scheme only got under way in March, but next year Suh (himself a former grant holder) is planning to put aside \$3-4 million for off-beat ideas, two per cent of his total budget. Because this is less than allowed tolerances on spending, the scheme has not been formally sanctioned by the directorate's advisory board, although according to Butcher most members know about it and approve. The high risk projects supported this year include a scanning optical microscope with a resolution of 50 micrometres, which could be used to examine living cell membranes, studies of polymerization in supercritical fluids and a proposal to generate "squeezed state light", which one reviewer described as being potentially as important as the discovery of the laser.

Butcher emphasizes that the new grant does not eliminate peer review: all proposals are still put through the normal assessment procedure, but some that get mixed reviews and so are rejected by programme managers are given a second chance by his committee. Only one in three of the proposals considered by the committee were funded this year, but Nam Suh hopes that as the word gets around that his directorate will support off-beat ideas, researchers will be encouraged to be more daring in the sorts of applications they make. **Tim Beardsley**

US gene cloning

Academics barred from P4 lab

Washington

THE National Institute of Allergy and Infectious Diseases (NIAID) is to ban the use by outsiders of its high containment laboratory at the Frederick Cancer Research Facility in Maryland. The laboratory, which was much used in oncogene research in the late 1970s, is one of only a handful of laboratories with P4 rating, the highest level of containment facility, and research being conducted there may have to be reclassified at a lower level of confinement if it is to continue elsewhere.

The Frederick laboratory, although formally run by and for the National Institutes of Health (NIH), has in practice often been used by outside researchers who needed access to secure containment. For the past year, the laboratory has been used successfully by Jack Murphy of Boston University Medical Center to clone fusion genes consisting of a synthetic oligonucleotide coding for human alpha-melanocyte stimulating hormone and portions of the structural gene for diphtheria toxin; the research could eventually lead to a "magic bullet" treatment for melanoma.

Last week Murphy went to the subcommittee on toxin cloning of the NIH Recombinant DNA Advisory Committee (RAC) to ask permission to continue the work at a lower level of containment, and also to seek approval for developing under P4 containment chemotherapeutic agents that would kill T and B cells carrying interleukin-2 receptors. This work, to be done in collaboration with Terry Strom of Beth Israel Hospital in Boston, might allow the creation of specific immune defects to prevent rejection in transplant pa-

tients, because resting and "memory" lymphocytes do not express interleukin-2 receptors.

Myron Levine of the University of Maryland, one of the most liberal members of the toxin subcommittee, said the proposed new work should be done only under P4 containment; other members agreed, noting that the effects of chronic exposure to such agents would be unpredictable and potentially harmful. But the subcommittee was then informed by Malcolm Martin, department chairman of the NIAID molecular biology laboratory, that Murphy could no longer use the Frederick facility because of the institute's decision to restrict access to NIH employees.

Faced with the option of preventing Murphy's work altogether, the subcommittee decided that the transplant rejection work could go ahead under P3 conditions, while the melanoma work could be reclassified as a hybrid between P2 and P3 until more toxicity data are gathered.

Martin explained later that the decision to exclude non-NIH workers had been taken because of competition from intramural research and an imminent refurbishment. There is another P4 laboratory in the NIH division of safety that is still available to outside researchers, although the space available is too small for many purposes. But Martin said that to require containment higher than P2 for Murphy's work "doesn't make any sense" because the host bacterium, the disabled K-12 strain of *Escherichia coli*, has no potential to colonize humans. **Tim Beardsley**

Indian guided missile nearly ready

New Delhi

THE Indian missile programme has reached a crucial stage and an advanced guided missile is expected to be ready as early as next year. Earlier this month, Prime Minister Rajiv Gandhi and Defence Minister P. V. Narasimha Rao saw the missile at the Defence Research and Development Laboratory (DRDL) at Hyderabad. They also watched the simulated flight of the indigenously developed missile and the static testing of its motors. Mr Gandhi later laid the foundation stone for a £75 million research facility at a 1,000-acre site 22 kilometres from Hyderabad where the missiles will be "assembled, tested and checked out". The new facility is scheduled to be fully operational in three years, but it is expected to show results next year.

No details are available about the missile's capability or range or if it can carry a nuclear payload. But the development has been welcomed in India in the light of Pakistan's nuclear ambitions. According

to defence scientists, it will have solid and liquid propellant motors and will be "highly sophisticated". The propellants, inertial guidance and control systems have all been developed indigenously.

The missile programme is directed by Mr Abdul Kalam, director of DRDL and chief designer of India's satellite launch vehicle, the workhorse of the Indian Space Research Organisation (ISRO). Four years ago, Kalam was brought from ISRO to DRDL to develop a surface-to-air missile for the defence system that depended exclusively on missiles imported from the Soviet Union.

DRDO, under the directorship of Dr V. S. Arunachalam, who is a metallurgist, is also working on electronic warfare, early warning systems, radars, submarines and a main battle tank (MBT) for the army. DRDO recently signed an agreement with Italy for collaboration on electronic counter-measure systems.

K. S. Jayaraman

UK research councils

The selling of NERC research

THE smallest source of Natural Environment Research Council (NERC) income casts a disproportionately large shadow. Private sector and overseas government contracts account for only £3-4 million out of a £94 million budget; yet, as NERC chairman Hugh Fish emphasizes, "bolting on some consideration of commercialization" is an important priority for NERC strategic research. These activities give Britain's brightest scientific lights overseas experience, which Mr Fish thinks important, and, more to the point, "it is in the national interest that research should be applied as quickly as possible to earn the country money".

What NERC is selling is expertise in applying research to specific technical problems; in contrast, the marketability of hardware that stands alone is of almost minimal interest. Such a priority points to the developing world as an obvious NERC market, one which many of its institutes

are now exploiting.

While some of this work is directly contracted with foreign governments, Mr John Morris, senior executive officer of NERC's marketing group, says that a preferred arrangement is a collaboration with a consultant group. The consultants are then responsible to the government or world-aid source for long-term monitoring, logistics and penalty clauses, leaving NERC only as a subcontractor of technical know-how.

The Institute of Terrestrial Ecology (ITE) is a NERC organization that has been involved in contract work for over 10 years. According to Professor Fred Last, ITE deputy director, ITE's experience with contracting has now given it the freedom to choose contracts that fit into its fundamental science programme. He is concerned, however, that few of these contracts have room for a supplement to support basic research; in real money terms, contracts have not yet made up for the decreases in the basic science budget, leading to a "burning up of the resources" on which contracts are based.

The organizational embodiment of NERC's commitment to marketing its strategic and applied research is its Research Marketing Group, which is divided into two parts. The first, which Mr Morris calls "science push", involves finding the NERC projects with exploitable applied research and then working out possible logistics, such as, for example, offering a commercial client the resources of several NERC institutes rather than a single project. The "demand-pull" side, on the other hand, keeps up with the priorities of world-aid funding agencies. Mr Morris claims that far from endangering NERC objectivity by private funding, NERC actually has an edge by offering companies what they do not want to hear as well as what they do. The research group, says Mr Morris, has not been around long enough to judge its effect in financial terms; but the group has had a clear influence in developing "an enthusiasm for a balanced push into commercialism" among NERC institutes.

Examples of this balanced push include efforts by the Institute of Marine Environmental Research to form a joint company for the assessment and exploitation of marine resources concentrating on low-level toxicity testing, marine surveys and mathematical modelling.

Another is the GLORIA sidescan sonar system for mapping the ocean floor, developed by the Institute of Oceanographic Sciences. In cooperation with the US Geological Survey, IOS has been using GLORIA to map the 200-mile economic exclusive zone off the west coast of the United States, with plans for this year covering the Gulf of Mexico and Puerto

Rico. As with other NERC marketing efforts, GLORIA is portrayed as essentially the selling of expertise.

A year ago, NERC, the largest single contractor for DG XII, the European Commission's directorate responsible for science research and development, set up a Brussels branch of its Research Marketing Group to liaise with the Commission.

Elizabeth Collins

British Association

Scottish meeting

BILLED as a festival of science, the annual meeting of the British Association for the Advancement of Science (BA) will be held on 26-30 August at the University of Strathclyde in Glasgow. Roughly 1,500 members are expected to attend, with day visitors and school parties bringing that number up to nearly 3,000.

Exploitation of research is the meeting's primary theme. Over half the papers offered will touch on this topic, including sessions on opportunities in British engineering, engineering in agriculture and the art of forestry. Applied science is also a year-round concern for the BA, as shown by its science and industry committee and increasing corporate interest in the organization. Corporate membership fees have risen more than fourfold over the past year, from £6,221 in 1983-84 to £25,850 in 1984-85.

Another principal theme of the meeting, waste disposal, will be covered by the BA debate on disposal of radioactive waste as well as papers on energy recovery from refuse incineration, geochemical aspects of acid rain, waste disposal and energy, and pollution in the sea. Those interested in the third theme, population, can attend lectures on the biosocial aspects of Scottish migration, breeding in the sea, urban policy issues, and contraception and the control of fertility.

Three BAYS (BA Young Scientists) lectures will be offered, on all-optical digital circuits and the future optical computer, vision and movement and superficial chemistry. Other activities have also been planned to interest the roughly 500 expected BAYS visitors to the meeting.

Sir George Porter will be elected president of the BA on the last day of the meeting. The BA budget has increased by roughly £34,000 this year, from £289,351 in 1983-84 to £324,330 in 1984-85.

Elizabeth Collins

Correction: New Zealand science

SPENDING on research and development in New Zealand in 1984-85 amounts to \$NZ200 million by government, \$NZ68 million by universities and \$150 million by industry. These figures were given incorrectly in the supplement on Science in Australasia (*Nature* 18 July, p.187). □

US troops and AIDS

Washington

THE US Department of Defense (DOD) will from 1 September require civilian blood banks to disclose the names of active-duty personnel whose blood tests positively for antibodies to the acquired immune deficiency syndrome (AIDS) virus. The blood banks have agreed only reluctantly to comply with the DOD request.

Blood banks such as the American Red Cross plan to inform potential military donors, before their blood is taken, that results of tests may be passed on to military authorities; details of how the policy will be implemented have yet to be settled. But the spectre of military donors refusing *en masse* to give blood as a result has become a source of anxiety for blood banks, some of which obtain a substantial proportion of their blood donations from servicemen.

DOD argues that knowing who has been exposed to the AIDS virus could be important for service readiness; for example, vaccinations given routinely to servicemen could be dangerous to those with immune deficiencies. Homosexual rights advocates, who represent one of the high-risk groups for AIDS, protest that unless kept confidential, information from AIDS antibody tests could be used to stigmatize or discriminate against those who have evidence of antibodies, even though many of these may not be infectious and only a small proportion are likely to develop overt disease. The link between AIDS and intravenous drug abuse might also lead to stigma. But DOD, which is separately considering its own mass screening of military personnel for AIDS antibodies, says that test results will be used only for medical evaluation and counselling.

Tim Beardsley

Japan to Halley

Second comet probe launched

Tokyo

AFTER several days of delay because of bad weather, Japan's main Halley's comet probe (Planet A) finally had a perfect launch from the Uchinoura Space Centre, Kagoshima, on the morning of Monday 19 August.

The satellite was carried aloft by a 28-m tall Mu 3SII solid-fuel rocket at 8.33 a.m. Some eight minutes later, each of the rocket's three stages had separated according to plan and the final "kick" stage had injected the satellite directly into its orbit. Test transmissions were picked up shortly afterwards by National Aeronautics and Space Administration (NASA) tracking stations in California and in Japan, indicating that the launch had been faultless.

The 135 kg satellite, given the name *Suisei* (Comet), will continue into orbit around the Sun. By March next year it will have completed three-quarters of its first revolution and rendezvous with Halley's comet soon after perihelion. Also on hand will be the Japanese satellite *Sakigake* (MST5), launched in January this year and at present orbiting the Sun, waiting for March.

In March, *Suisei* will pass within a million kilometres of Halley's comet and take a series of vacuum ultraviolet (Lyman alpha line) images of the comet's coma as it passes perihelion. This stage of the comet's journey is of particular interest, for water and other volatile materials leave the comet nucleus and are photodissociated through a complex series of intermediate radicals. The UV images should make it possible to identify exactly what stages are involved.

Suisei is rather small compared with the satellites from other nations that will be observing Halley, and everything possible has been done to save weight. The satellite has not been fully stabilized by a three-dimensional attitude control system but will rotate slowly at 5 revolutions per minute. Each time ultraviolet pictures are taken, the satellite will be briefly slowed down by a momentum wheel. A sharp picture will then be ensured by rotating the image on a charge-coupled device to compensate exactly for remaining spin.

Also on board is a pair of sensors for electrons and positive ions intended to observe the interaction of the comet's ionosphere with the solar wind — and particularly to look for turbulence produced by solar flares. Measurements will be coordinated with those from *Sakigake* which will be sitting further "downwind". Although it was launched largely to test the new control and communication subsystems now in use on *Suisei*, it also carried instruments to measure plasma waves and the interplanetary magnetic field.

The launch of *Suisei* is by far the biggest

adventure so far for the Institute of Space and Astronautical Science, the independent academic organization responsible for all Japan's scientific satellites. Preparations have been under way for some

years: a new rocket had to be developed with extra strap-on boosters, a new launch tower built at Uchinoura Space Centre and a new 64-m diameter dish antenna built in Nagano prefecture to pick up the signals. If *Suisei* proves to be a success, a lunar satellite may come next on the list of Japan's ventures into space.

Alun Anderson

University committee review begins

THE promised scrutiny of the British University Grants Committee (UGC) is to be carried out by a committee under the chairmanship of Lord Croham (formerly Sir Douglas Allen), chairman of the British National Oil Corporation; the deputy chairman of the committee is Lord Butterworth, retiring vice-chancellor of the University of Warwick.

The development is important because it is almost certain to lead to a change in the constitution of UGC, founded in 1919 as a buffer between the British government and the publicly supported universities. The need for such a review was most recently advocated by the Jarrett committee on the efficiency of British universities, which reported earlier this year.

The composition of the review commit-

tee is not what British academics would have chosen. Lord Butterworth, made a life peer at the beginning of the year, has been a successful vice-chancellor. The committee also includes Dr Paul Matthews, vice-chancellor of the University of Bath until two years ago, and Mr John Smith, secretary of Imperial College, London. Dr Peter Clarke, chairman of the Scottish Vocational Education Council, was previously principal of Robert Gordon's Institute of Technology at Aberdeen.

The other members of the committee are primarily industrialists, including Sir Alex Alexander, chairman of J. Lyons and Co., Mrs Mark Baker, a director of Thames TV Ltd, Sir Austin Bide, chairman of Glaxo Ltd and Mr Ian Beesley, head of the Prime Minister's Efficiency Unit. □

Soviet science

In the steps of the Ukraine

THE Ukrainian Academy of Sciences, the largest and oldest in the Soviet Union except for the All-Union academy itself, seems rapidly to be becoming a model for the other thirteen republics. During the past few months, the Soviet media have given considerable acclaim to the Ukrainian system of "production centres", semi-pilot factories operating under the academy's aegis. And last month *Pravda* lauded another major Ukrainian innovation — its network of local science centres.

Science centres have been a major feature of Soviet science since the establishment of the Siberian branch of the Soviet Academy of Sciences in 1957. Except for the Ukraine, however, science centres have largely been confined to the Russian republic where, since there is no separate Russian academy, they have come under the All-Union academy.

In spite of official encouragement of science centres as a means of bridging the Soviet Union's endemic gap between research and implementation, the centres have often been criticized for failing to fulfil their allotted tasks. A campaign in the mid-1970s began with the Far Eastern Science Centre and worked its way up to the Siberian branch of the academy before the tide turned.

The Ukrainian network began formally with the establishment, in 1971, of five centres, for the Donetsk, Western, North-Eastern, Dnieper and Southern regions of the Ukraine. By 1980, more than 25 per

cent of the whole scientific work-force of the academy was employed in regional centres, spanning marine biology and cryomedicine, high-molecular chemistry and radiophysics. Major developments in the chemical and instrument-building industries have been credited to the centres.

How far the Ukrainian experience could be repeated in other republics, however, is not clear. The Ukrainian SSR is one of the most developed republics of the Union, with large reserves of ores and fossil fuels, as well as much of the best agricultural land. The science centres clearly have had the support of the First Secretary of CPU, V.V. Shcherbytsky, who some years ago publicly urged that Ukrainian scientists should concentrate first and foremost on research needed for the Ukrainian economy. Moreover, the Ukrainian republic is large enough, and has an economy diverse enough, to provide employment for a wide range of scientific talent.

The main problem of the Ukrainian centres as of science in all the non-Russian republics is that of attracting top-flight people. Job prospects in the Soviet Union depend not so much on what, but where, one studies. The brightest students from all republics gravitate towards the top higher education institutions in Moscow and then, almost inevitably, become absorbed into the Moscow-Leningrad-Novosibirsk research network.

Vera Rich

French nuclear tests

Mystery of *Rainbow Warrior*

LAST month's sinking in New Zealand of *Rainbow Warrior*, flagship of the Greenpeace environmental organization, and the killing of the ship's photographer, have stirred a murky pot of accusation and counter-accusation in France. The French press has taken the scandal to its heart.

One of the more speculative magazines, *VSD*, has even gone so far as to accuse the Elysée palace (the French White House) of having organized the attack to demonstrate French power in the Pacific and determination to continue nuclear weapons testing on Mururoa Atoll; all might have gone well, the theory goes, had not a mole from the British secret service, MI5, buried within its French equivalent, DGSE, tipped off the New Zealand authorities (somewhat too late, it must be assumed). The Elysée will be suing *VSD*,

I was told that Limpet Mines
had no fall-out....



but other speculations implicate KGB, CIA, the home-grown French extreme right and other supposed enemies of equilibrium.

In the more serious French press, a picture is emerging that the two people held and now formally accused in New Zealand are probably members of the French Army seconded to DGSE to survey the activities of Greenpeace. It is suggested that the frogmen who attached two limpet mines to *Rainbow Warrior* left behind their French inflatable dinghy and bubble-free breathing apparatus (stamped "Made in France") and probably escaped in a hired yacht, *Ouvea*, belonged to a separate French group.

The bombers themselves, like *Ouvea*, may have come from New Caledonia, the group of French Pacific islands which is fast becoming President Mitterrand's Algeria — a country peopled by some 91,000 native Kanaks seeking independence and wooed by M. Mitterrand but also 54,000 French colonists who are determined the island shall stay French. These French might have wished to discredit the present government — and hence the trail of evidence left behind, it is suggested.

However, this relatively convenient picture is inconsistent with the fact that

Ouvea was hired (for FF 70,000 cash) through a Paris agency. The hirers were an adventurist French doctor (who specializes in diving medicine, but who left *Ouvea* before the attack and claims he knew nothing of the object of the mission) and three more shadowy figures, since disappeared with the yacht.

The *Rainbow Warrior* crew also believes there was a French "mole" aboard, a woman who sent articles for a "French paper" and one of whose maps of New Zealand waters was later found on *Ouvea* (when the yacht was briefly stopped and then released, to the later chagrin of the New Zealand police). This woman has also disappeared, via, apparently, an archaeological dig in Israel.

President Mitterrand has asked M. Bernard Tricot to carry out an investigation and to inform the public.

But if indeed the French secret service does prove deeply involved, what possible government motive — or perception by junior officials of a government motive — could there have been to bomb *Rainbow Warrior*? The only possibility could be to protect the French nuclear weapons tests on Mururoa, not far from New Caledonia

and Tahiti, which Greenpeace intended to disrupt. The French nuclear arsenal is in continual evolution and even expansion with a postulated introduction of "enhanced radiation weapons" or neutron bombs and a testing campaign of at least another 15 years is planned. Speculation by the French that *Rainbow Warrior* was carrying sophisticated devices for detecting neutron bombs (discounted by Greenpeace, which says the ship carried only a geiger counter and apparatus for relaying pictures to news agencies) suggests that perhaps France was indeed planning a neutron weapon testing programme.

But whatever the merits of this speculation and of an attack on Greenpeace as a counter-measure, the opposition of South Pacific states to French testing, at the rate of 60 weapons between 1977 and 1984, is now liable to increase, since the signing earlier this month by seven nations of a declaration that the South Pacific should be a "nuclear free zone". The treaty, which forbids signatories to build or test nuclear weapons, will be ratified by the end of the year, Australian Prime Minister Bob Hawke has predicted, and though powerless to prevent French testing in the region it will certainly increase French political isolation. The *Rainbow Warrior* affair can only have done the same.

Robert Walgate

French mistrusted on Mururoa

MURUROA Atoll, according to New Zealand and Australian scientists who visited the French nuclear weapons testing site in October 1983, is likely to release its accumulated load of debris from underground explosions to the Pacific "within 500–1,000 years".

At present, they say, emissions from the island and consequent fallout in the South Pacific region are having a negligible effect on health and they do not suggest that even the likely release of the contained radiation within a few centuries will be "consequential". But the scientists' evident suspicion of French calculations about the security of the site is symptomatic of the South Pacific's distaste for what the French are doing with "their" atoll.

For example, "French information on venting is very qualitative", the South Pacific scientists say in their report, "and there is no reason to believe their figure that more than 99 per cent of the radioactivity is retained in vitrified material". And any "implicit impression" that venting is barely detectable is "almost certainly false".

On underground containment, the report indicates that the French tests are "probably" 700–1,200 m below the bottom of the lagoon, in the volcanic base of the atoll, which is surmounted by 180–500 m thickness of limestone. But the limestones contain some highly porous sections and the volcanics are fractured for 400 m

around each explosion. "The volcanics in their virgin state offer a poor to moderate geochemical barrier and a moderate to good hydrological barrier. The testing programme is reducing the effectiveness of both", the report says. Moreover, "the claim that the transition zone [between the volcanics and the porous limestone] acts as a barrier to long-term leakage can, on the basis of geological evidence, be discounted".

Thus there will be leakage within 500–1,000 years, in which transuranic elements without a particular geochemical affinity (such as neptunium-237) may be expected to be leached out. "It would be surprising if this leakage were consequential", the report says, but such scientific assurances, according to an Australian government spokesman last week, have made very little impression on public opinion. Rational or irrational, the public view is that far more atomic weapons have now been exploded in the South Pacific than in the north (the public thinks of Hiroshima and Nagasaki) and that it is simply high time the bombing stopped.

But France will not stop, which may, in the long run, lead to a search for a new site. A deal with French Africa to use the Sahara (from which the testers decamped to Mururoa in 1966), or a deal with the United States to use its own sites, emerge as the only faint possibilities.

Robert Walgate

Mt Wilson Observatory

SIR—We have recently returned from what seems all too likely to be our last observing run as guests of the Carnegie Institution with the 100-inch telescope at Mt Wilson, California. We had 14 consecutive nights on the telescope. All of them were clear; the only cloud that we saw during that whole time was a little cirrus, which did not impede us at all, on one night. Star image diameters near one second of arc were the norm, and on several occasions we enjoyed “seeing” much better than that — the remarkable seeing for which Mt Wilson is renowned.

To experience such enviable observing conditions for such a sustained period on our final observing run heightens the irony of Mt Wilson Observatory being closed down ostensibly because of the deficiencies of the site. The 100-inch was shut down finally, as far as its owner the Carnegie Institution is concerned, on 26 June 1985.

Like every one of the major instruments on Mt Wilson, the 100-inch has played a role of extraordinary distinction and significance in the progress of astronomy throughout much of this century. But it should not now be allowed to rest on its laurels. The same telescope that served Hale and Adams, Hubble and Humason, Baade, Sandage and countless others so well in the past is still in the same class as the very best of the newer telescopes, and behind its now-closed shutters it sits ready and waiting to resume the battle with the pressing problems of present-day astronomy. In this connection it is of interest to recall that observers at the 100-inch, casually viewing Saturn, were well aware of the “gramophone-record” appearance of the rings¹ before *Voyager* drew attention to that structure; but an experienced planetary observer who observed Saturn under the best conditions at the 200-inch Palomar telescope specifically asserted² that no subdivisions exist in the rings.

The very short time-scale upon which the announcement (made barely a year ago) of the closure of Mt Wilson is being implemented has made it impossible for any other organization yet to assume financial and operational responsibility for the observatory. Although the 100-inch telescope is the first to close, the 60-inch and the solar telescopes will soon follow it if they too are not taken over by other operators. The formidable researches that are being undertaken with those instruments, all of them long-term projects that would suffer severely from even a temporary interruption, are described with extraordinary honesty in the current annual reports of both the president of the Carnegie Institution and the director of the Mt Wilson & Las Campanas Observatories. The juxtaposition in those reports of the current achievements of Mt Wilson Observatory and the closure of the self-

same place leaves the reader altogether incredulous.

The Athay Committee, which was set up at the request of the director of Mt Wilson to advise on the future of the observatory, rightly refers to the “anguish” of astronomers around the world at the closure of such a distinguished observatory. The committee’s report makes a strong case — actually not a difficult thing to do — for the retention of Mt Wilson in operation, and includes a recommendation that Carnegie should at least continue to run the observatory for a reasonable period until an orderly takeover by another operator can be arranged. Unfortunately, the Carnegie Institution has apparently not been able to counter reasoned argument with reasoned argument, but instead has chosen to respond with ever fiercer press releases that appear to constitute its only acknowledgement of the receipt of the report which it requested. Its position seems analogous to that of St Paul on the Damascus road before his conversion³; we devoutly hope that the parallel is completed and/or that some other institution will soon be found or set up to maintain and even enhance this great observatory. Long live Mt Wilson!

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*The Observatories,
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Soviet tests

SIR—If your reporter meant to imply in his report on the American Association for the Advancement of Science (AAAS) meeting in Los Angeles¹, that I accused the US government of lying on the question of Soviet compliance with the Yield Threshold Test Ban Treaty (YTTBT), I must take exception. I personally have no way of knowing for certain whether the assertions by some representatives of the US government^{2,4} that the Soviet Union has tested over the treaty threshold of 150 kton are accurate, even less do I have any grounds for implying intent to deceive on the part of those representatives.

What was pointed out at the AAAS meeting is that the techniques for calculating unbiased estimates of the yield of underground nuclear tests have been well understood for over a decade^{5,6}. The most recent published work that I am aware of⁷ concludes that within the resolution of the seismic data and analysis techniques used, the Soviet Union has not significantly exceeded the 150 kton limit. Unlike most questions of Soviet treaty compliance, this work does not depend upon classified data or analysis techniques. If the US Depart-

ment of Defense (DOD) has reason to believe that this work is in error, it has a right and an obligation to demonstrate that in the open literature.

However, recent reports indicate^{4,8} that work within DOD supports the finding that the Soviet Union is complying with the terms of YTTBT. If these reports are correct, DOD should make the information public. Surely no-one can have forgotten the bitter lessons of the past 20 years; in a free democratic society, the bond of trust between the government and the people is also a vital component of national security. Moreover it is unthinkable that responsible people would let false pride over a small technical error contribute to making matters any worse than they already are between the two great nuclear powers.

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Prehistoric calendar

SIR—Correspondence has recently revealed a mistake of 1° in the azimuth given for the equinoctial sunset notch at the prehistoric site at Brainport Bay, Argyll (*Nature* **314**, 158; 1985). The reduction of six solar observations taken at the Oak Bank stone backsight gave an average azimuth of the notch of 99.46° west of true north and its declination of +0° 8' was calculated from this. Somehow this azimuth became a true bearing of 261° 32.5' in the text and in Fig. 4, whereas it should of course be 260° 32.5'. A fresh reduction of the same observations by a different method gives the same azimuth and a declination of +0° 9.6'.

On 21 March 1985, about 26 hours after the astronomical equinox, the setting Sun was observed from the Oak Bank rock carving until it was about a solar diameter above the horizon, where it was photographed; at that point it disappeared into a bank of cloud but the disk was clearly heading directly into the notch. This of course confirms the calculations and also the inference drawn from them that we have at Brainport Bay a precise and still functioning equinoctial alignment.

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The science of sea dumping

SIR—In their letter on the science of sea dumping (*Nature* 11 July, p.100), Alan Reddish and Steve Cousins state that "scientific modelling should clearly specify the model in terms of the underlying theoretical assumptions". We agree, which is why the forthcoming Nuclear Energy Agency (NEA) report on the review of the continued suitability of the north-east Atlantic dump site¹ contains over 60 pages of description of the models developed by the Ministry of Agriculture, Fisheries and Food (MAFF) and the National Radiological Protection Board (NRPB) to assess the radiological impact of sea dumping of radioactive waste on both humans and marine organisms.

Clearly, Professor Holliday and his team² could not include all this detail in their report and there was no need since it is to be published elsewhere. However, the full description was provided to them, to the NEA expert group for the dump site review and to the panel of scientists carrying out the London Dumping Convention review.

Reddish and Cousins also express concern about experimental validation of models, but they fail to realize the difficulties involved and the extent to which field measurements have in any case been used. Direct validation of the whole suite of models is simply not possible because of the large time and space scales involved and the very low radionuclide concentrations. What can be done is to validate those parts of the models that have most effect on predicted radiological impact.

Studies of model sensitivity show clearly that it is the interactions between radionuclides and suspended and seabed sediments that most influence doses. This part of the model has been validated¹, using data on naturally occurring radionuclides in the ocean. Data from the earlier dumping site in the English Channel cannot be used for direct validation because the site is coastal while the models deal with dumping in the deep ocean and because monitoring has failed to detect any radioactivity that can be traced to this site. Irish Sea data are also coastal and so could not be used for direct validation, but they can be, and were, used in fixing some model parameters, particularly the factors by which coastal water organisms concentrate radionuclides.

One method of telling whether the results of a particular modelling approach are robust is to tackle the problem in a conceptually different way. The basic model used to estimate dose to man was first of all to predict the distribution of radionuclides in space and time, using an oceanographic model and then to calculate the expected concentrations of radionuclides in sea foods by the application factors.

The concentration factors anticipate the concentration of radionuclides in surface and mid-water marine organisms, relative to the ambient water, as a result of the accumulation of radionuclides from all sources including the ingestion of food. The values used are, for the most part, derived from direct observations. The sea foods eaten by man are all taken in coastal and surface waters to depths of up to 1,500 m.

No sustained food chains from the deep sea (> 4 km) to man are known but, as a safety measure, calculations were also made of the dose to man that could arise if fish living at the dump site itself, close to the bottom, were directly consumed by man. All these calculations indicate very low expected dose rates to man at daily consumption rates of considerable quantities of sea food.

The conceptually-different approach used in the sensitivity analysis was to estimate the dose to man that might arise if, by some chance, contaminated organisms within the dump site were consumed by organisms from outside the area, resulting in radionuclides eventually being transferred to man at concentrations that would not be predictable from the ambient water concentrations, and over a much shorter time scale.

The results of making a number of calculations, based on various ideas as to how such a transfer of radionuclides could in theory be effected, led to the conclusion that even if such occurrences do take place, the resulting dose to man would still be exceedingly low.

There is often a common misconception as to what, exactly, models are attempting to achieve. In this case it is an attempt to estimate the dose to man, and the impact on the deep-sea fauna, as a consequence of disposing packaged radioactive waste into the deep sea. The models used do not aim to represent in detail all known geochemical, oceanographic and biological processes. What they do attempt is a distillation of the major processes and parameters that would be primarily concerned in estimating the dose to man and, by the application of sensitivity analyses model-to-model comparisons, and a verification of major parameters by environmental observation, produce sufficient information for decisions to be made.

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1. NEA Review of the Continued Suitability of the Dumping Site for Radioactive Waste in the North East Atlantic (Nuclear Energy Agency, OECD, Paris, in the press).
2. F.G.T. Holliday Report of the Independent Review of Disposal of Radioactive Waste in the Northeast Atlantic (HMSO, London, 1984).

Pain and animals

SIR—R. Sharpe's letter (*Nature* 25 July, p. 290) contains a sentence beginning "Since it is impossible to devise a method of measuring pain in animals it follows that...". If this premise was true, it would indeed follow that regulations against cruelty to animals would depend on doubtful subjective judgements. The problem is not how to assess an animal grossly disturbed by some acute injury which no humane human would allow to continue. Veterinarians and scientists are faced with the much more difficult problem of judging animals with a chronic disorder such as arthritis.

A number of groups have proposed solutions and in particular the International Association for the Study of Pain has published its obligatory guidelines (*Pain* 16, 109–110; 1983). They require a two-stage investigation. First the animal in question is compared with normal animals by measuring quantitatively its behaviour such as movement, grooming, sleep etc. and by measuring its physiology such as EEG, hormone levels, autonomic state and so on. The animal is then treated with a relevant analgesic procedure and its behaviour and physiology are again measured. The first measure describes the abnormality of the animal in objective terms and the second measures the degree of abnormality attributable to pain. It is as crucial for animals as for babies that we should use every available skill to measure their pain in spite of the fact that we cannot speak to them.

P.D. WALL

(Managing editor, *Pain*)

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Improving crops

SIR—Close to this town lives a tribe of Indians who practise a clearly defined method for improving their crops, particularly maize. Every family plants its own seed, avoiding contamination with alien germplasm. When they find an outstanding example, they do not use it for food or seed; they give it as a present to a friend, who is not supposed to eat it or use it in any other way, but to plant it. The whole action is treated as a religious ceremony or social convention, presumably part of a system ensuring the tribe's continuity. The exchange involves a large part of the community, and is made in all directions.

The obvious objective is to increase the frequency of favourable genes with additive actions, by selecting within a mildly inbred population, in which new forms with desirable characteristics are introduced periodically.

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Selenium not for dumping

California is up in arms because of selenium in some drinking water. Does it not know that selenium is also an essential dietary supplement?

WHY do so many land animals depend so crucially on inorganic substances predominantly present only in sea water, selenium for example? The question has most recently been provoked by the fuss, in California, about the discovery last year of selenium in the water stored in the Kesterson reservoir. Officials of the city of Monterey have since refused to allow that supposedly toxic fluid to be allowed to return to the Pacific Ocean. In their ignorance, they have overlooked a question that is not merely of practical importance (how do we keep our people healthy?) but one that has profound significance for people everywhere — where did we come from?

This is how the tale goes. Life began in the oceans or shallow seas. The primitive enzyme systems depended on inorganic ions. (Physiologists have often remarked on the resemblance between body fluids and sea water.) But some of these vital inorganic substances are needed in only small amounts. These are the nutritionally essential trace minerals, such as iodine, molybdenum, manganese and zinc. When living organisms emerged from the oceans, animals and plants that became land-dwellers had to find soil that supplied these minerals. Iodine is uncommon except near the oceans. Plants do not need it, but goitre became an endemic disease of animals in inland areas lacking iodine.

Geological variations in the Earth's crust, glaciation during the great Ice Age, the effects of vulcanism and the results of erosion and leaching are some of the factors that decide which parts of the Earth's surface are either deficient or oversupplied with trace minerals.

In some parts of the world, most conspicuously in Australia, the soil is so deficient in certain trace minerals such as zinc and copper that plants will not grow. Elsewhere, soil contains a toxic excess of some minerals. One of these is selenium, which was known for many years only as being poisonous, even carcinogenic. It came into prominence because certain plants such as *Astragalus* (locoweed) concentrate it from high-selenium soils so that horses that eat *Astragalus* may become poisoned, or "locoed" ("blind staggers"). In the 1930s, Franke and his co-workers reported effects of "toxic wheat" grown in South Dakota on high-selenium soil.

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Feeding toxic wheat or selenium to hens produced deformed embryos in their eggs with eyes and beaks missing, and with distorted wings and feet¹. No such injuries were detected in human beings; the only bad effects noted were dental caries.

The bad reputation of selenium was abruptly changed in 1957, when it was found in laboratory studies to be a nutritionally essential trace element for rats, chickens and pigs^{2,4}. This news was followed with startling rapidity by reports of selenium deficiency in farm animals all over the world, especially in New Zealand. Selenium provided one of the most dramatic illustrations of the rule of Paracelsus that "the dose alone determines the poison", a rule that seems to have been forgotten by most of us.

Among the many studies of the toxicity of selenium were some carried out by the US Food and Drug Administration (FDA) which led to the conclusion that high levels of selenium are carcinogenic⁵. Since official thinking does not permit the extension of Paracelsus' rule to carcinogens, FDA agonized for 15 years before it allowed selenium to be added to the diet of farm animals.

Ironically, selenium had meanwhile emerged as an anticarcinogen and as a component of an important enzyme, glutathione peroxidase, an enzyme important in the metabolism of injurious hydroperoxides⁶.

Now move to China, where multiple myocarditis, "Keshan disease", is found in children in populations with low levels of blood selenium living in low-selenium areas. Epidemiological studies show an inverse correlation between blood selenium and age-adjusted total cancer death rates and liver cancer incidence. In areas with high selenium levels, there is significantly lower mortality from cancer of the oesophagus and stomach. As a public health measure, a programme has been started of spraying grain crops with sodium selenite solution⁷. The difficulty may be that both the population and the food supply may be less mobile than in the United States.

In California (where anything can happen, and usually does), selenium leaching from the coastal foothills of central California (perhaps a result of acid rain) got into irrigation water and became concentrated in drainage reservoirs. Aquatic birds, coots and wild duck, living in the Kesterson reservoir, re-enacted in

1984 the 1930 drama of the South Dakota chickens, producing deformed offspring. Environmentalists sprang into action, saying that selenium would probably cause deformed human embryos. The headlines flared. Officials in Monterey acted. A former head of California Water Resources Control Board spoke of the "Kesterson nightmare", and predicted⁸ "another year's crop of what may very well be contaminated produce".

In reality, the "contaminated produce" is probably helping to protect consumers against cancer^{9,10}. The permissible level of selenium in drinking water, set by the Environmental Protection Agency (EPA) at 10 parts per thousand million, should probably be raised to 50 to 100 parts per thousand million as a public health measure.

Until FDA gave approval for selenium as an animal feed additive, farmers literally had to return to the ocean, the original source of selenium for animals, to obtain selenium for their livestock. This was done by feeding fishmeal, the selenium in which was natural and therefore legal. Selenium has another role in ocean fish; it prevents the injurious action of mercury that is naturally present in seawater and is added to the ocean by volcanic discharge and run-off from coastal soils.

Various hearings on the disposition of the Kesterson water have brought forth recommendations ranging from stopping agriculture in the vicinity of Kesterson to skimming off the surface soil and burying it. In none of the hearings has the essential nature of selenium, or the fact that selenium is a natural resource that tends to be depleted and lost, been discussed or even mentioned. Nor have tolerable levels of intake been suggested. The objective seems to be to treat selenium as a toxicant that must be sequestered, yet much of the land in California and more in Oregon, is deficient in selenium. Shall we have to go back to the sea?

Thomas Jukes

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Sodium pump

Birthday present for digitalis

from D. G. Allen, D. A. Eisner and S. C. Wray

EXACTLY two hundred years ago, William Withering published his monograph on the therapeutic use of the foxglove *Digitalis purpurea* in congestive heart failure, then known as dropsy¹. Digitalis is known to bind to the ($\text{Na}^+ + \text{K}^+$) ATPase, the enzyme that acts as the Na^+/K^+ pump in the membrane of cells, and in consequence inhibits the extrusion of Na^+ and the inward movement of K^+ ions². The publication, elsewhere in this issue, of the amino-acid sequence of the α -subunit of the ($\text{Na}^+ + \text{K}^+$) ATPase^{3,4} makes a fitting celebration of the bicentenary. But knowing more about the structure of the Na^+/K^+ pump and the way in which cardiac glycosides may affect it should not be allowed to obscure the uncomfortable fact that inhibition of Na^+ and K^+ movements may not be the sole or even the most important therapeutic action of the cardiac glycosides.

Although cardiac glycosides, such as digitalis and ouabain, are now among the most commonly used cardiac drugs, many aspects of their mechanism of action and their clinical use are still controversial. Three questions of pharmacological interest are currently claiming attention: how the cardiac glycosides increase the force of heart-muscle contraction (positive inotropy); whether there are naturally occurring cardiac glycosides in human plasma; and whether the cardiac glycosides are equally valuable in all types of heart failure. Recent work has suggested that these issues may be related and may lead to a better understanding of the way in which digitalis works.

The conventional view of the positive inotropic mechanism is shown in the figure (refs 5,6). Briefly, digitalis inhibits the Na^+/K^+ pump, leading to an elevation in the intracellular Na^+ concentration, which acts on a membrane $\text{Na}^+/\text{Ca}^{2+}$ exchange to increase the intracellular calcium concentration; this increases the Ca^{2+} content of the sarcoplasmic reticulum and leads to release of Ca^{2+} during systole. There is good experimental support for the increase in the intracellular Ca^{2+} concentration, which represents the final stage of this process⁷. Support for this scheme comes from the fact that other manipulations that inhibit the Na^+/K^+ pump also increase the force of contraction. Under these conditions, there is a close relationship between internal Na^+ concentration and force⁸. This hypothesis has, however, been criticized on two grounds⁹. First the plasma concentration of cardiac glycosides in patients is about 10 nM whereas Na^+/K^+ pump inhibition, at least *in vitro*, is only seen at a concentration of about 100 nM (ref. 9). Second, low

concentrations of cardiac glycosides have been reported to stimulate rather than inhibit the Na^+/K^+ pump in various isolated preparations, as shown by measuring either ATPase activity¹⁰ or internal Na^+ concentration¹¹.

Interpretation is complicated because it has been suggested that the apparent pump stimulation is secondary to release of catecholamines from nerve endings¹². This criticism cannot, however, be levelled against the demonstration that the isolated ATPase is stimulated by cardiac glycosides¹³. These experiments have also shown that there is a maintained increase of activity that is not further increased by low doses of glycosides if the ATPase is first washed with a salt solution. This suggests that the stimulation might result from displacement of an endogenous

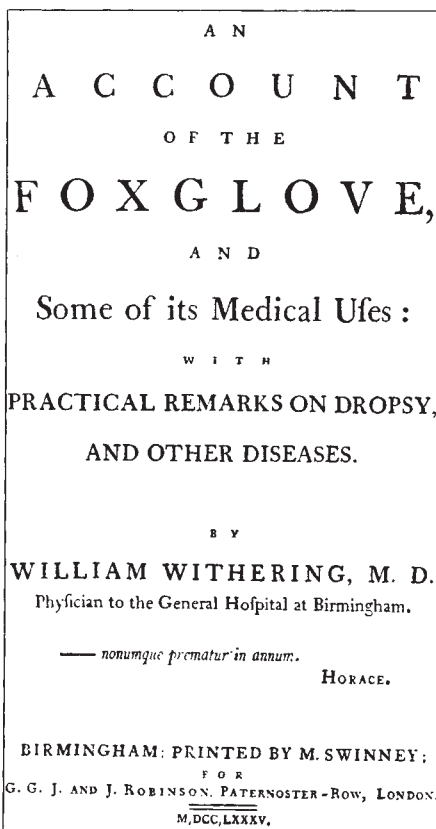
actually inhibiting the Na^+/K^+ pump. The major unresolved question of the 'non-inhibition' hypotheses is how glycoside binding can increase force. Possibly binding may interfere with Ca^{2+} bound to the surface membrane¹⁵. Another intriguing experimental finding is that intracellular injection of low concentrations of glycosides can lead to an inotropic effect that is independent of binding to the extracellular Na^+ pump site¹⁶. Despite these problems with the Na^+/K^+ inhibition hypothesis, however, no clear alternative has yet been proposed.

There is much interest in the possibility that the cardiac glycoside receptor on the Na^+/K^+ pump was 'designed' to bind an endogenous glycoside-like substance present in human plasma. As Na^+/K^+ pumps are found in a wide variety of tissues, such an endogenous substance would cause, amongst other effects, increase in cardiac contractility, constriction of blood vessels and an increase in Na^+ excretion by the kidney. Endogenous Na^+/K^+ pump inhibitors have been found in toad skin¹⁷ and rat hypothalamus¹⁸, and a substance that inhibits the Na^+/K^+ pump has recently been isolated from the blood of hypertensive patients¹⁹. This is particularly interesting because it may be a cause of essential hypertension. Release of this substance in response to a plasma Na^+ load could increase Na^+ excretion by the kidney; possibly it is the elusive natriuretic hormone²⁰. This substance should, however, also inhibit the Na^+/K^+ pump in vascular smooth muscle, so producing vasoconstriction and consequent increase in blood pressure.

Information about the structure of the Na^+/K^+ pump may help us to understand how it is affected by glycoside binding. The sequence data allow us to identify the parts of the molecule that lie within the membrane and those that protrude into the extracellular and intracellular spaces. The ATP binding site and the phosphorylation site are also revealed, and preliminary suggestions can be made about the position of the glycoside binding site. It is particularly instructive in this regard to compare the ($\text{Na}^+ + \text{K}^+$) ATPase sequence with that of the Ca^{2+} -ATPase, also published in this issue²¹. Ca^{2+} -ATPase does not bind glycosides and the molecule lacks the sequence that Schull *et al.*³ suggest is the ouabain binding site on the ($\text{Na}^+ + \text{K}^+$) ATPase.

One of the first questions is how glycoside binding leads to inhibition of the ATPase and to the movement of ions, but more detailed knowledge of the binding site and possible interactions with other regions of the molecule may suggest further consequences of glycoside binding that could underlie the therapeutic effects. For example, glycoside binding might lead to a conformational change in the molecule, thus affecting Ca^{2+} -binding in the surrounding membrane.

The amino-acid sequences and structural information so far published show



inhibitor (see below). One problem with this hypothesis is that simply displacing one inhibitor by another at the same site should not result in a stimulation of the pump.

In this context, it is interesting that recent measurements of ouabain binding to membranes have shown two classes of binding sites^{11,14}. The lower affinity one may be the conventional site at which the Na^+/K^+ pump is inhibited, whereas the high affinity one might provide an alternative positive inotropic mechanism without

that the Na^+/K^+ pumps from the electroplax organ of *Torpedo californica*¹ and the kidney of sheep² are strikingly similar. Both α -subunits have 1021 or 1022 amino acids, about 87% of which are homologous, and there are runs of 128 and 108 amino acids that are identical. Six regions of 22–29 hydrophobic residues are probably big enough to span the membrane and the intracellular regions of the molecule can be identified from the locations of the ATP binding site and the phosphorylated site. It is assumed that the glycoside binding site(s) lie on one of the three regions that protrude into the extracellular space. Assuming the glycoside binding site can be unequivocally identified in future, it will be of considerable interest to see to what extent the glycoside binding sites are conserved in pumps from different tissues and species and, in particular, to compare species that have very different sensitivities to cardiac glycosides. Homologies between this and other ion-pumping ATPases^{3,4,21} are substantially lower but offer the intriguing possibility of identifying the crucial amino-acid sequences for common functions, such as ATP binding and energy transduction.

At present cardiac glycosides are used clinically to increase cardiac output in two major conditions. In atrial fibrillation, where the increase in heart rate renders the heart inefficient as a pump, the glycosides, through their action on the vagus nerve, slow the heart and so increase its output. They are also used to treat heart failure, where the rate is normal, but the efficacy of digitalis under these conditions is controversial. Although digitalis treatment can produce a small, short-term benefit²², controlled trials have found little evidence of benefit from maintained digitalis therapy. This may, of course, simply mean that the initial treatment allowed

the heart to recover. Alternatively, prolonged exposure to glycosides could result in the synthesis of more Na^+/K^+ pumps²³, so compensating for those inhibited by the glycosides. If there is an endogenous glycoside-like substance that competes with the therapeutic glycosides for the inhibitory site on the sodium pump, it will be necessary to monitor its concentration during treatment before we will understand the therapeutic role of glycosides.

Whereas, in isolated preparations, large doses of glycosides lead to a substantial inotropic effect that can be accounted for by the mechanism shown in the figure, at the low doses used therapeutically the inotropic effect is much less marked. Even so, it leads to a small but worthwhile clinical improvement. The magnitude of this low dose effect may well depend on the presence of endogenous factors that compete for the same binding site. □

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Astrochemistry

Chemistry in dynamically evolving interstellar clouds

from David A. Williams

RECENT observations of interstellar molecular clouds have shown that they have a great diversity in density, temperature and chemical state. The study of these clouds has become important in astronomy because it is generally believed that they are the progenitors of stars. In an important theoretical contribution, Tarafdar *et al.* have described a framework within which the diversity of the clouds may be understood. They suggest that the observed variety of conditions and molecular state is a natural outcome of the evolution of the clouds, as they contract under gravity from low density atomic gas to high density molecular material.

Chemistry in interstellar clouds is now a well established subject² and the mathematical modelling of extensive reaction networks has been investigated by many authors during the last fifteen years. These studies have shown that whereas low density clouds are generally in a chemical steady state, time for denser clouds to approach a steady state — typically 30 million years (ref. 3) — is probably greater than the age of the clouds. It is unlikely, therefore, that dense clouds have achieved a chemical steady state, in spite of the apparent success of some steady state models⁴. A time-dependent approach is necessary, especially if one assumes that molecules in the gas collide and stick to dust grains and are not returned to the gas. If this is the case, a

steady state can never be achieved. The time required before this process seriously depletes molecules from the gas is about $10^5/n$ years, where n is the density of hydrogen atoms per cubic centimetre. This time is obviously quite short when the density is high, say $n > 10^4 \text{ cm}^{-3}$. Time-dependent models of the chemistry of static clouds^{3,5}, however, also fall short in some significant respects when compared with the observations, and the models clearly require further development.

The latest refinement to be included in the models depends on the recognition of the dynamical character of interstellar clouds. The spectral line widths observed from the clouds are several km s^{-1} , much broader than would be expected (about 0.1 km s^{-1}) for gas at rest at the temperatures of interstellar clouds, indicating there must be considerable motion in the gas. Therefore, turbulence, low velocity shocks, expansion or contractions must be driving the gas to supersonic velocities. For example, J.E. Dyson, T.W. Hartquist and D.A. Williams (ref. 6 and in preparation) have developed a model of giant molecular clouds in which continuous low-mass star formation creates fast winds that drive the bulk motions and erode dense clumps of gas⁷, maintaining the chemistry permanently out of steady state.

In an alternative approach, Tarafdar *et al.*¹ have considered an isolated, spherical, initially low density cloud. For gravita-

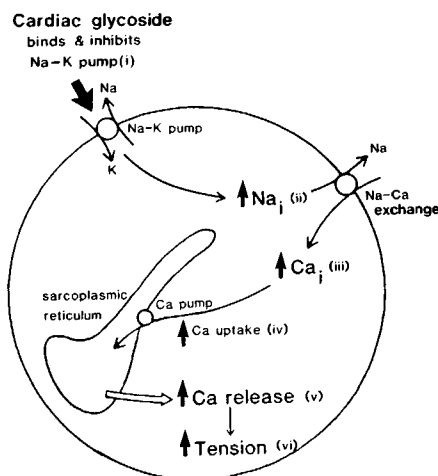


Diagram of a cardiac muscle cell, showing the conventional view of the positive inotropic action of the cardiac glycosides. Binding inhibits the Na^+/K^+ pump (i), elevating the intracellular Na^+ concentration (Na_i , iii) and thence intracellular Ca^{2+} (Ca_i , iii). This leads to an increase in the Ca^{2+} content of the sarcoplasmic reticulum (iv), with increased release of Ca^{2+} (v) and increase in tension (vi).

tional collapse of the cloud to occur, its thermal and turbulent pressure must be insufficient to hold up the cloud against gravity. The temperature structure of a cloud is therefore of paramount importance and Tarafdar *et al.*¹ have carefully determined the temperature profile of low density clouds. They have shown that such clouds are indeed gravitationally unstable and will collapse, without the need to involve a trigger of shocks or thermal instabilities, as had been suggested by earlier workers. They consider that this collapse explains the observed large line widths. They follow the collapse numerically, using a hydrodynamic computer code developed by Bodenheimer⁸, and simultaneously follow the evolving chemistry in the gas, using a restricted version of the reaction network described by Prasad and Huntress⁵. In addition, the description indicates the loss of molecules by sticking to grains. Their results show that the extinction at the cloud centre increases as the collapse proceeds, and the molecules are freed from the major loss caused by photodissociation, with the result that their abundance rapidly build up.

The models of Tarafdar *et al.* provide some very interesting conclusions. In all cases, regardless of the starting conditions, central densities attain very high values ($n > 10^6 \text{ cm}^{-3}$) within a few million years. A few percent of the cloud mass is in the form of this high density clump at the cloud centre. If these clumps proceed to form stars, a plausible rate of star formation for the Galaxy can be derived.

The chemical condition of the gas is also fully explored. Because the gas spends much of its time in a low density state, the predicted molecular abundances are dramatically affected. The excellent success of the model in describing the chemistry may be judged from the figure, which shows how the fraction of gas in the form of CO varies with extinction, A_v , along the cloud diameter (in this model, extinction is merely another measure of elapsed time in the contraction). A high C-atom abundance is also derived from these models. Observations of other atoms and molecules are also plausibly encompassed in this description. Thus, the observed differences are merely snapshots at different

instants during the continuing collapse of clouds from a diffuse state to some dense state, from which — presumably — stars are formed. The initial diffuse clouds are all predicted to be fairly similar.

Several questions deserves further study: for example, will the high C-atom abundance drive the formation of unacceptably high hydrocarbon densities? Do clouds really evolve as rapidly as suggested? If stars do form, will the cloud remnant be entirely disrupted? Is all high density material in the form of transient clumps? The answers to such questions will surely be forthcoming. Tarafdar and colleagues have opened up a very

promising avenue in the study of the interstellar medium and star formation. □

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Cellular immunology

Suppressor T cells in the network

from N. A. Mitchison

MOST of the elements of any complex control system look inwards at one another — in the immune response most lymphocytes interact with one another rather than with the outside world, just as neurones do in the nervous system. Within the regulatory compartment of the immune system the major components are helper and suppressor T cells (Th and Ts cells) which control the activity of the cytolytic T cells and antibody-producing B cells of the effector compartment. Helper T cells act directly on these effector cells but suppressor T cells usually act indirectly, via inhibition of helper T cells. It is this inhibitory interaction between suppressor and helper T cells that is analysed by two papers on pages 738 and 741 of this issue^{1,2}.

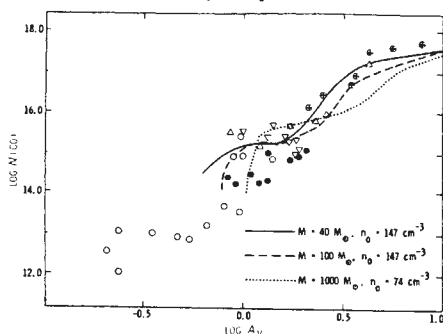
The question asked is who recognizes whom: do Ts recognize Th, or is it the other way round? This sounds simple enough, but it is made complicated by the dual nature of the receptor that T cells bear. It recognizes both antigen and a MHC (major histocompatibility complex) molecule, a property valuable to T cells because it prevents them from interacting with free antigen and ensures that they react only when it contacts other cells.

This obviously matters for cells like T cells, which operate only at short range, and is not important for B cells which secrete long-range antibody molecules. Tada has termed interactions of T cells with other cells as both 'linked' and 'cognate'. Linkage occurs via an antigen bridge connecting the antigen-binding parts of receptors on the two cells, and cognate binding of the T-cell receptor to an MHC molecule occurs on the partner cell ('cognate' means that receptors can recognize the MHC molecule as a result of repertoire selection in the thymus). In the Th-Ts interaction the 'link' must be symmetrical, that is, receptors on both cells bind to separate parts of a single antigenic structure. The direction of recognition is determined by the cognate part of the in-

teraction: if the receptor on the Th cell binds to an MHC molecule on the Ts cell, then it is the Th cell that is doing the recognizing, or vice versa.

An additional possibility is that receptor idotype (a structure on the variable part of the receptor) can substitute for antigen, in which case the linkage involves simply one receptor binding directly to another. Note that this type of binding is entirely symmetrical, because in such an interaction one cannot in principle distinguish between the idiotype structure and the receptor that binds to it.

During the past five years two substantial studies seemed to have answered the 'who-recognizes-whom' question^{3,4}: both reached the conclusion that the Ts cell contributes an MHC molecule and is therefore the partner which is recognized. It releases this molecule as a single chain, linked by a disulphide bridge to a second antigen-binding chain, which in turn establishes an antigen bridge with the Th cell. There was still some uncertainty as to whether the Th cell used its standard receptor (the 'Ti' receptor, which it uses to interact with antigen-presenting cells and B cells) for this purpose. That is where the I-J molecule, or determinant, comes in. Klein *et al.*³ argue that the Th cell does use its standard receptor, and that the I-J structure which distinguishes Ts cells from other lymphocytes is simply a modification of an MHC class II molecule — perhaps a set of determinants unique to an isolated (monomeric) MHC chain as suggested by Bodmer.⁴ Despite these small differences, at that time there was general agreement about the major conclusion, and a substantial series of experiments seemed to confirm predictions made by the 'Th-recognizes-Ts' hypothesis. In addition, the hypothesis seemed to fit with the growing evidence for 'veto' cells, antigen-presenting cells within the thymus which also inactivate any Th cells that recognizes them.



Comparison of theoretical and observed variation in $N(\text{CO})$ with A_v . Modified from ref. 2, where references to the original data sets shown by the various symbols are given.

These suggestions are all contradicted by the experiments reported in this issue^{1,2}, which show quite clearly that the recognition properties of Ts cells are determined not by the MHC molecules which they bear, but by selection of their receptor repertoire in the thymus. Thus, lymphocytes from allogenic chimaeras can make the soluble factor with suppressor activity (TsF) of host type even though they do not bear host MHC molecules (they can also make donor-type TsF, presumably because both donor and host MHC molecules are present during repertoire selection in the thymus). The conclusion is that what was previously regarded as an MHC molecule should now be reinterpreted as an anti-idiotypic which binds to that part of the Th cell receptor that recognizes MHC molecules.

Much of the data of Klein *et al.* do not contradict this view (see ref. 3 for review). All that is needed is the substitution of an anti-idiotypic of the required type whenever an MHC molecule was identified previously as a restriction element.

Note that the concern expressed by Uracz *et al.*² about expression of I-J on macrophages must be misplaced: host macrophages are presumably needed to elicit idiotypes characteristic of the host MHC-binding site on Th cells that in turn permit TsF to express anti-host idiotypic binding activity.

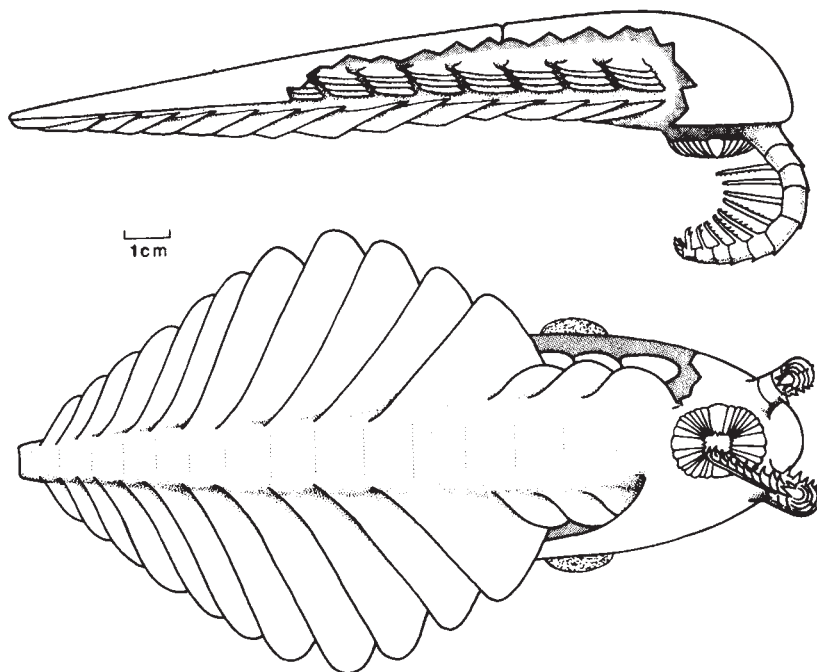
Other possibilities exist but they all seem to involve discarding even more experimental data. Meanwhile we are still waiting for crucial molecular information, particularly about the difference between the mouse strains used to raise anti-I-J antibodies, and about the relative molecular mass 33,000 peptide that is precipitated by these antibodies³. If the interpretation offered in the two new reports^{1,2} is correct, the peptide must be part of the Ts cell receptor for antigen + MHC.

There is nothing new for cellular immunology in having to reinterpret quite large chunks of data; that has already happened, for instance, during the elucidation of IR genes. No doubt it will do Ts cells no good at a time when they are, for other reasons, running into heavy weather⁶. Nevertheless, these important papers from Japan must be regarded as a major achievement for the idiotypic network theory, and they will strengthen the view already current⁶ that T cells are even more strongly subject to network control than are B cells. □

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A Cambrian enigma



Reconstructed lateral and ventral views of *Anomalocaris nathorsti*, appendage partly flexed, lateral lobes in still position. In the lateral view, a portion of external cuticle is cut away to show the arrangements of gills. For the ventral view, part of head region cuticle is cut away to show flaps and gills, and blades are omitted from the right appendage to expose the circling of mouth plates. (From *Phil. Trans. R. Soc.* **B309**, 569; 1985).

How well do we know the fossil record? Recently H.B. Wittington and D.E.G. Briggs (*Phil. Trans. R. Soc.* **B309**, 569; 1985) have described in detail yet another surprise from that almost inexhaustible palaeontological treasure-house, the Middle Cambrian Burgess Shale of British Columbia (see *Nature News & Views* 302, 570; 1983 and 314, 403; 1985). *Anomalocaris*, the bizarre metazoan shown above has had a chequered scientific history; its isolated jointed appendages have been known for many years from both the Lower and Middle Cambrian of North America. They were generally regarded as the detached limbs of a large arthropod. With the discovery of complete specimens, however, a far more bizarre and enigmatic creature has emerged, with two varieties presently recognized (species or sexual dimorphs?). With a length of probably up to half a metre, it is the largest known Cambrian animal. The body was flanked by flexible lobes that served in propulsion, while gills were housed beneath lateral extensions. A single pair of appendages arose from the underside of the head to flank a remarkable jaw that consisted of a series of plates ending in sharp prongs that encircled the mouth. Although the gut contents are not identifiable, the evidence points to *Anomalocaris* being a predator.

Whittington and Briggs' account is important for our understanding of early metazoan life. Until recently, most palaeontologists believed Cambrian predators rare, exerting a negligible influence in marine communities, although some

years ago G.E. Hutchinson (*Am. Ass. Adv. Sci. Publ.* **67**, 85; 1961) suggested that this was an artefact of the fossil record that favours suspension and deposit feeders with robust skeletons (Schopf, T.J.M. *Paleobiology* **4**, 261; 1978). *Anomalocaris* is the latest, if most ferocious, addition to the lightly skeletized and soft-bodied predators that have been found in the Burgess Shale (Conway Morris, S. *Phil. Trans. R. Soc.* **B 307**, 507; 1985). At some horizons that have yielded *Anomalocaris* there are also trilobites showing evidence of unsuccessful attack, and Whittington and Briggs speculate on how the action of the jaw apparatus can be reconciled with the damage. But some injured trilobites are not associated with *Anomalocaris* and perhaps represent the unwelcome attention of other large predators that so far have eluded discovery (Conway Morris, S. & Jenkins, R.J.F. *Alcheringa* **9**; in the press).

The Burgess Shale contains an extraordinary variety of metazoans which, if found alive today, would probably be hailed as new phyla. *Anomalocaris* is no exception; despite the arthropod-like anterior appendages, the rest of the anatomy is irreconcilable with the arthropod bodyplan and the jaw apparatus appears to have no parallel in the Metazoa. It is a reminder of how little we know about the early diversification and ecology of the metazoans.

Simon Conway Morris

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Cell biology

Yeast as the universal cell

from Ira Herskowitz

"IN ten years, yeast has changed from *E. coli* with a nucleus to the universal cell." This comment by Lee Hartwell (University of Washington) opened a recent conference of yeast cell biology*, reflecting the two main recurring themes of the meeting. The first was that yeast shares with animal cells many of the molecules and processes that are the objects of intensive study in modern cell biology. It has, for example, ubiquitin, calmodulin, clathrin, endocytosis, peptide growth factors, small nuclear RNAs, retroviral elements and those staples of cell structure and function, actin and tubulin. Thus, such central problems as protein localization, function and synthesis of the mitotic apparatus, and the workings of the cell cycle can all be productively studied in yeast. The second theme was that just about any biochemical, cytological or genetic technique or experimental strategy can be applied in studies with yeast. What distinguishes it from other eukaryotic cells for studying cell biology, however, is its accessibility to the full range of both traditional and modern genetic methods. The power of these techniques to elucidate such fundamental processes as mitosis, endocytosis and the control of the cell cycle was well illustrated at the meeting.

The most powerful of the modern genetic strategies available is to produce mutations in cloned yeast genes *in vitro* and then to replace the wildtype gene with the mutant by recombination¹ *in vivo*. This makes it possible to link biochemical information on a protein *in vitro* with cytological information on structures *in vivo* by studying the behaviour of the mutant. A model analysis of this sort has been carried out by G. Payne (University of California, Berkeley) on clathrin, which is in coated vesicles² and is believed to mediate endocytosis. An antiserum to purified clathrin allowed the gene encoding the heavy chain of the protein (*CHC1*) to be fished out from a library, disabled by mutation *in vitro* and then replaced in yeast. The first result with the *in vitro* clathrin mutant (*chc1*) is that it is, perhaps surprisingly, viable, although the colonies it forms are small. Further studies should help to define the role played by clathrin in yeast. Are the mutants defective in endocytosis, in cell fusion events (such as occur in mating), or in turnover of cell surface material?

The mitotic apparatus

Much work in cell biology is focused on how the mitotic apparatus is assembled and disassembled during the cell cycle and

how it distributes chromosomes to daughter nuclei. An initial goal is to define the proteins that make up the spindle on which the chromosomes become arrayed and then to determine how they function³.

The yeast spindle is a tripartite structure, consisting of microtubules that join at one end to the centromere (the yeast version of the kinetochore of the chromosome) and at the other end to the microtubule organizing centre, the spindle pole body (analogous to the centromere)⁴. The molecular outlines of the yeast mitotic apparatus are now emerging through information from the following sources.

The central element of the structure is the microtubule, composed of tubulin. The story described by D. Botstein (Massachusetts Institute of Technology) for the budding yeast *Saccharomyces cerevisiae* and by M. Yanagida (Tokyo University) for the fission yeast *Schizosaccharomyces pombe* is the same: there are three tubulin (*TUB*) genes, two of them for alpha tubulin (only one of which is essential) and one for beta tubulin. Microtubules are needed for chromosome distribution: inactivating an essential tubulin gene by mutation has the same consequences as adding the popular anti-mitotic agents, nocodazole and benomyl. A suggestion that microtubules might also be involved in bud growth seems not to be borne out, as mutants lacking an essential tubulin gene nonetheless form buds. A crucial question in the mechanism of mitosis is that of the role of the microtubule-associated proteins, which are oft-speculated to play prominent roles in governing microtubule association and dissociation. They have now been identified in yeast by traditional fractionation and polymerization (F. Solomon, Massachusetts Institute of Technology), and the analysis of mutants produced by gene replacement should quickly establish what part they play.

The most exciting findings so far have been on two genes that affect the spindle pole body (P. Baum, University of Washington). It has long been hoped that some of the numerous *CDC* (cell division cycle) genes⁵ would affect structural components of the segregation apparatus, and it looks as though this might be the case for *CDC31*. The nucleotide sequence of *CDC31* shows that it has homologies to calmodulin and in particular that it contains calcium binding domains. The *CDC31* protein may therefore be a structural component of the spindle pole body with its properties of association controlled by calcium. The second fascinating gene described by Baum seems to control the number of spindle pole bodies. Remarkably, mutants defective in this gene form

extra spindle pole bodies when the *ESPI* protein is inactive — up to eight can be seen by electron microscopy. The *ESPI* gene product thus seems to stop spindle pole body assembly after each round of their duplication at each cell cycle.

Studies of the *KARI* gene indicate that it may also code for a structural component of the spindle pole body and also demonstrate a novel strategy for the use of cloned genes to generate mutant phenotypes. M. Rose (Massachusetts Institute of Technology) observed that overproduction of the *KARI* protein (achieved by placing the *KARI* gene under control by a regulated, highly expressed promoter) has lethal consequences similar to those of inactivation of the *KARI* product. In both cases there is a block in chromosome distribution. This strategy of creating a mutant phenotype by overproduction of a functional product ought to be useful in mammalian cells. It was suggested that a high level of the *KARI* protein might disrupt spindle function by 'tying up' an essential spindle component. The original mutant allele of the *KARI* gene has a distinct phenotype — nuclei fail to fuse after mating between compatible cells. Such defects have been suggested to be due to a defect in the spindle pole body^{6,7}.

Although the mitotic spindle is a symmetrical structure (at least on the cell-biological drawing board), the *ndc1* mutant described by Botstein may reveal ways in which it is not. These mutants display the remarkable aberration that in some cases the daughter cell receives both sets of chromosomes and in other cases the mother cell receives both sets. (It is perhaps relevant that *cdc31* mutants can exhibit similar behaviour⁸.) This aberration in chromosome distribution suggests that one half of the mitotic spindle is defective in some manner. Perhaps the *NDC1* protein is a connector between the spindle pole body and microtubules. One would have to make the additional interesting speculation that the two spindle pole bodies differ from each other. Localization of the *KARI* and *NDC1* (as well as *CDC31* and *ESPI*) proteins within the spindle by direct cytological observation will be a critical test of these speculations. *ESPI*, *CDC31*, *KARI*, and *NDC1* are notable examples in which genetic analysis has turned up interesting mutants but whose further characterization relies on cytological methods that are newly available in yeast^{9,10}.

The cell cycle

Yeast genetics has traditionally been a major contributor to the elucidation of the control of the cell cycle, defining the genes necessary for the initiation, progression and exit of cells from the growth cycle to various specialized shunts, such as meiosis. The *CDC* genes, which control progression, have recently attracted particular attention because of the possibility that they may have functional homologies to

*The Cetus-UCLA Symposium on Yeast Cell Biology, was held in Keystone, Colorado, USA, 9-14 April 1985.

cellular oncogenes of animal cells¹¹. There was therefore considerable interest in a report at the meeting from S. Reed (University of California, Santa Barbara) on *CDC28*, mutations that cause arrest in G₁ without any inhibition of protein synthesis, by contrast with numerous other mutations and with nutritional starvation that result in arrest in G₁ without protein synthesis and are thus less likely to reflect specific controls. The nucleotide sequences of both *CDC28* from *S. cerevisiae*¹² and the functionally related gene *CDC2* of *S. pombe*¹³ show clear homology to protein kinases and Reed reported that the *CDC28* product has protein kinase activity¹⁴. Defining its targets may thus provide insights into key regulators of the cell cycle.

Mating between *S. cerevisiae* cells of opposite mating types, *a* or α , involves reciprocal arrests of the mating partners in G₁ by oligopeptide pheromones, *a*-factor and α -factor. Understanding how these agents arrest the cell cycle provides a novel opportunity to study regulation of the cell cycle. Some of the components of this system have now been identified. The working view is that each factor binds to a receptor on the cell surface and then generates an intracellular signal that ultimately leads to arrest. The cells then go on to fuse. The *STE2* gene appears to be a component of the receptor for α -factor (D. Jenness, University of Washington), and the *STE3* gene has been argued to be a component of the receptor to *a*-factor. The nucleotide sequences of *STE2* (A. Burkholder, University of Washington) and *STE3* (E. Jarvis, University of Oregon) show that both code for several long hydrophobic stretches and hence exhibit hallmarks of membrane proteins. Do *STE2* and *STE3* proteins make up channels of some sort? Only the appropriate receptor components are produced in *a* and α cells because the *STE2* gene is expressed only in *a* cells and the *STE3* gene only in α cells, due to the action of regulatory proteins coded by the yeast mating type locus¹⁵. Evidence that response to *a*-factor and α -factor proceeds by a common intracellular signal was presented by G. McCaffrey (University of Oregon) in experiments using α mutants that produce the receptor normally found on *a* cells.

Regulators involved in the decision to leave the mitotic cycle and enter the pathway of meiosis and spore formation have been identified. Prior genetic analysis pointed to the *RANI*¹⁶ product of *S. pombe* and the *RME1*¹⁷ product of *S. cerevisiae* as such regulators. Recent analysis in my laboratory confirms that *RME1* governs entry into meiosis. In particular, the *RME1* product appears to be an inhibitor of meiosis and sporulation: these processes occur only in cells whose *RME1* gene is turned off (which occurs naturally in *a*/ α diploid cells). Determining the manner by which the *RME1* and *RANI* products act

(as a repressor or a protease?) and identifying their targets, ought to provide insights into the early steps of meiosis.

The stage seems set to learn a great deal from yeast about fundamental cellular processes that have historically been considered within the domain of cell biology. Yeast geneticists no longer flinch at cell

biologist's jargon: clathrin, tubulin, ubiquitin — while cell biologists are learning a new three-letter code: *TUB*, *CDC*, *ESP*. This meeting, the first devoted to yeast cell biology, was well timed to show that contemporary cell biology and yeast research, both areas of great excitement, are joined for a productive future. □

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Ichthyology

Filter-feeding on a grand scale

from Jared M. Diamond

ON 29 November 1984 a second specimen of the megamouth shark *Megachasma pelagios* was captured near Los Angeles, California¹. The original specimen caught off Hawaii in 1976 and possibly the most significant discovery of a marine vertebrate for a decade, constituted not only a new species and genus but also a new family within the shark order Lamniformes^{2,3}. Of particular interest are megamouth's feeding habits, which may be unique among animals and on which the Los Angeles specimen sheds further light.

Among the over 300 specimens of sharks, *Megachasma pelagios* is one of only three that are filter-feeders; the others are macropredators and scavengers. Filter-feeders strain many small food items out of water or mud either by pumping the medium through a stationary filter, as in the case of sessile invertebrates such as bivalve molluscs or polychaete worms, or by pushing the filter through the

medium, as do flamingoes and many fish. The secret of successful filter-feeding lies in filtering large volumes of medium containing high concentrations of food items. Among filter-feeders are the largest mammals and fish — baleen whales, basking sharks, whale sharks and manta rays — for which the problems of filtering enough food are acute. These large filter-feeders are constrained to live at or near the surface of nutrient-rich waters, such as coastal zones and the Antarctic.

Megamouth, which at 4.5 m in length and 750 kg in weight is one of the largest living sharks, is clearly a filter-feeder. The stomach of the Hawaiian specimen contained 1-inch long euphasiid shrimps; shrimps and deep-water jellyfish were found in the Los Angeles specimen. The shark's huge mouth is filled with hundreds of tiny teeth, none over 0.5 inches high. Its highly protrusible jaws can be extended like a hoop-net. Filtering is carried out by a unique type of gill-raker, with a core of cartilage and covered by tiny denticles.

In habitat and anatomy, megamouth is very different from the other two large filter-feeding sharks. The basking shark swims with its mouth open, near the surface of cold nutrient-rich coastal waters. It is a powerful swimmer, with a streamlined body, strongly calcified skeleton, stiff fins, strong skin, and firm muscle and connective tissue. Its filtering apparatus consists of vast gill cavities and streamlined gill-rakers, designed to support a high flow rate. The whale shark is also a strong swimmer that often feeds near the surface and can suck in and filter out water even when stationary.

By contrast, megamouth lives in deep water with lower plankton concentrations

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Megamouth shark *Megachasma pelagios* captured off Los Angeles in November 1984 (Photograph courtesy of L. J. V. Compagno).

than surface waters; one specimen was taken at 125 feet or less, the other at about 540 feet. The soft loose skin, flabby muscles and connective tissue, soft cartilaginous skeleton with little calcification, and non-streamlined form suggest it is a slow, weak swimmer, and explains why megamouth is the only shark known to be attacked by the 'cookie-cutter shark', *Isistius brasiliensis*, so-called because of the neat pieces it bites out of large fish and porpoises¹. Its inefficient filtering mechanism, with a small gill cavity, restricted internal gill apertures and non-streamlined gillrakers, is ill-suited for a high flow rate. Instead, it has a huge tongue that almost fills the mouth when closed, presumably to expel water across the filter with jaws shut.

How could such a large but weak animal, with a low-flow filter and living well below the rich plankton zone, filter enough plankton to support itself? A clue comes from the bright silvery lining of the mouth, suggesting that it is bioluminescent. The stomach contents include species that live in the deep scattering layer and that are known to be bioluminescent. Thus, megamouth may feed by holding its mouth open in the dark, attracting plankton like moths to a light trap. Some abyssal anglerfish have bioluminescent organs to attract their fish prey, but I am unaware

of a filter-feeder other than megamouth that uses light to attract plankton.

The closest parallel to megamouth's suggested feeding strategy may be found in frogmouths (family Podargidae), which are large nocturnal birds of New Guinea and Australia. Although frogmouths are well known to pounce on large insects and small vertebrates, this foraging technique fails to explain why they have a huge mouth. However, *Podargus papuensis* of New Guinea spends much time perched with its mouth wide open, perhaps using an odour to attract aerial plankton on to the sticky paste on its palate. If evidence is forthcoming to support the suggestion that megamouth sharks and frogmouths attract plankton into their open mouths by light and chemicals, respectively, the infrequency with which such behaviour has evolved will be puzzling. Why do almost all filter-feeders go to great efforts to pump water through their filters or to push their filters through water, instead of attracting their prey into their mouths? □

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Atherosclerosis

Scavenger cell receptor shared

from Michael S. Brown and Joseph L. Goldstein

WHEN a blood vessel is damaged, scavenger cells (macrophages) migrate into the wound where they ingest and degrade the debris that has escaped from the bloodstream. But problems with the normal process can arise within the walls of arteries, because the macrophages ingest, among other things, the cholesterol-carrying low density lipoproteins (LDL) of plasma that have penetrated into the artery wall. The insoluble cholesterol of LDL cannot rapidly be excreted and accumulates in the macrophages as lipid droplets, converting the macrophages into 'foam cells' that contribute substantially to the mass of atherosclerotic plaques¹. Our understanding of atherosclerosis would be advanced if we knew how macrophages identify abnormal lipoproteins and other debris for ingestion in preference to normal extracellular components. Answers are emerging from studies of a cell surface receptor on macrophages that binds chemically altered proteins and on page 746 of this issue, Phillips and colleagues show that platelets release a substance that affects this receptor².

Surprisingly, normal macrophages express very few LDL receptors of the classic type that is found on fibroblasts and liver cells³. They therefore ingest native

LDL sparingly. When LDL is, however, modified chemically by acetylation, it loses the ability to bind to the classic LDL receptor, but is now recognized by another high-affinity receptor on macrophages³. The receptor-mediated uptake of acetyl-LDL leads to massive deposition of cholesterol within the macrophage^{1,3}. The receptor that mediates this uptake has been called the acetyl-LDL receptor or the scavenger cell receptor¹. It also recognizes LDL and other proteins which have been treated with agents that alter lysine residues, including succinic anhydride, maleic anhydride and malondialdehyde^{1,4}. As with acetylation, these agents increase the net negative charge of the protein. Other negatively charged compounds, such as sulphated glycosaminoglycans also bind to it. Gotto and coworkers have recently solubilized and partially purified the acetyl-LDL receptor from the mouse macrophage cell line P388D₁. By ligand blotting, they have shown it to be a protein of approximate relative molecular mass (M_r) 260,000 (ref. 5), clearly different from the 160,000 M_r LDL receptor.

Although the acetyl-LDL receptor lacks absolute chemical specificity, it is cell specific. It seems to be present only on

macrophages¹ and endothelial cells^{6,7}. Endothelial cells are not usually considered to be scavenger cells because they show little phagocytosis of particulate matter⁸, but the discovery that they have acetyl-LDL receptors resurrects the possibility that they share some scavenger functions with macrophages. In the body the acetyl-LDL receptor is particularly abundant on the endothelial cells that line the hepatic sinusoids. When fluorescent or radioactive acetyl-LDL or acetoacetyl-LDL is administered to animals intravenously it rapidly accumulates in the hepatic sinusoidal endothelium and to a lesser extent in hepatic Kupffer cells⁹⁻¹¹.

There remains a crucial question — the identification of the natural ligand for the acetyl-LDL receptor. It seems unlikely that lysine residues would be acetylated in extracellular fluid *in vivo*. On the other hand, Steinberg *et al.* have observed that LDL can be converted by chemical oxidation into a negatively charged form that binds to the acetyl-LDL receptor¹². When LDL accumulates in extravascular spaces, it might undergo oxidation, thereby increasing its net negative charge, as well as permitting uptake by the acetyl-LDL receptor of macrophages. Such a mechanism might explain the occurrence of cholesterol-loaded foam cells in atherosclerotic plaques, and also the accumulation of cholesterol in macrophages throughout the bodies of patients with homozygous familial hypercholesterolaemia. Indirect support for this hypothesis comes from the finding that LDL isolated from inflammatory tissues of rabbits has an enhanced negative charge¹³.

Phillips *et al.*² add a new twist to the story. They show that activated platelets release a substance of $M_r > 100,000$ that blocks the uptake and degradation of acetyl-LDL by macrophages, apparently as a result of competitive binding. This substance is likely to be one or more of the negatively charged proteoglycans that are stored in platelet granules and released after thrombin treatment¹⁴. Secretion of this substance might limit the ability of macrophages to clear lipoproteins from extracellular deposits. In addition, this

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platelet-derived substance might serve as a chemotactic agent to attract blood monocytes to sites of tissue injury and to activate macrophages at these sites by interaction with the acetyl-LDL receptor.

The emerging story suggests that the acetyl-LDL receptor may mediate scavenger functions that are shared by sinusoidal endothelial cells and macrophages. At present, however, there is no direct evidence to link this receptor with cholesterol accumulation in atherosclerotic plaques. Possibly the physiological

ligand for this receptor is not a lipoprotein at all but some other negatively charged macromolecule, such as the one released from platelet granules². The receptor is now being purified³, so it should soon be possible to prepare antibodies against it, thus helping to remove some of the mystery surrounding its function. □

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Oncogenic intelligence

Another link in the chain

from Peter Newmark

IT CAME as a complete surprise when one chain of platelet derived growth factor was revealed to be very similar to that of the product of the *sis* oncogene and, again, when the epidermal growth factor receptor sequence was found to contain the sequence of the *erb-B* oncogene product. But, if the sequence of the *fms* oncogene product turns out to be identical to part of that of the receptor for the growth factor CSF-1, it will be no surprise, following the publication by C.J. Sherr *et al.* of highly suggestive evidence of a close relationship between the two proteins in *Cell* **49**, 656; 1985. This forging of a third link between the genes for growth factors or their receptors and oncogenes will add impetus to the quest to discover whether the mutation of the factor or receptor genes is a contributory cause of human cancer.

Of the two proteins compared by Sherr *et al.*, more is known of *fms*. Like most oncogene products, it was discovered in an animal tumour virus, in this case the McDonough strain of feline sarcoma virus. A good deal has been learnt by Scherr's group about the properties of both the viral protein, which is encoded by the *fms* oncogene (*v-fms*), and the protein that is encoded by the *fms* proto-oncogene (*c-fms*) — a gene of mammalian cells and the progenitor of *v-fms*. Less is known of the functions of either protein. The oncogene product obviously transforms normal cells into tumour cells. It also comes into the category of oncogene proteins that are located on the cell surface and are associated with an enigmatic ability to catalyse the phosphorylation of tyrosine residues in proteins; a similar enzymatic activity is associated with the product of the proto-oncogene. Particularly by analogy with *erb-B*, these facts have suggested that *c-fms* encodes a growth factor receptor and that *v-fms* encodes part thereof but with a truncation or other alteration that subverts the normal function of the receptor.

But which receptor is it? The probable answer has come from studies by E.R. Stanley's group of the receptor for CSF-1

(colony stimulating factor-1), which is a protein that stimulates the production of macrophages from their precursor cells. The factor binds specifically to cells of the mononuclear phagocytic series, including macrophages, and seems to have some characteristics that are reminiscent of the *c-fms* product.

To cement the relationship between their two proteins, Sherr's and Stanley's groups have now obtained two sets of data, which they jointly report in *Cell*. The first establishes the presence of a *c-fms*

product with an associated tyrosine kinase on the membrane of macrophages, as it should be if it is the CSF-1 receptor. The second demonstrates that antibodies to the *v-fms* product recognize a macrophage membrane protein that binds CSF-1 and has other properties of CSF-1 receptor.

Now we need sequence data. The sequences of *v-fms* and, by deduction, its product are known, but those of *c-fms* and the CSF-1 receptor are not. Only when they become available will it be possible to be certain that the *c-fms* gene encodes the CSF-1 receptor and to see what alterations have accompanied the conversion of *c-fms* into *v-fms*. As Sherr *et al.* say, there are two possibilities. One is that, parallel to the *v-erb-B* gene, *v-fms* may lack the sequence that encodes the CSF-1 binding domain of the receptor and could encode a tyrosine phosphorylating activity that is constitutively active rather than CSF-1 stimulated. But since the *v-fms* product does not seem to be seriously truncated, Sherr *et al.* make the alternative suggestion that the *v-fms* product is a competent receptor and that its delivery by tumour virus to cells that do not normally contain the receptor, particularly fibroblasts, provides them with an abnormal ability to respond to CSF-1, which fibroblasts themselves synthesize. □

Peter Newmark is Deputy Editor of Nature.

Particle physics

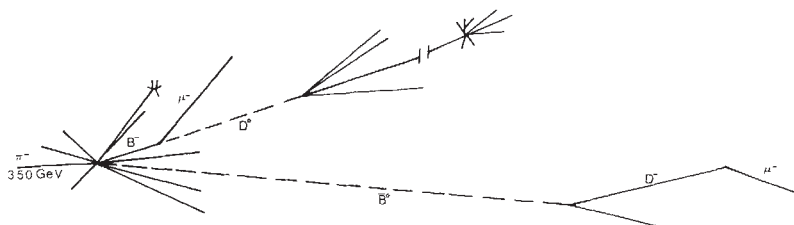
Naked beauty seen at last

from David J. Miller

CLEAR tracks of 'beauty' particles have at last been seen. The existence of the beauty or bottom quark has been accepted ever since the discovery of the upsilon particles in 1977, but the upsilons are the 'hidden beauty' bound states of a beauty quark with a beauty anti-quark. There should also be 'naked beauty' states in which the beauty quark binds with one of the lighter 'up', 'down' or 'strange' anti-quarks, forming B mesons. There has been evidence for some time for the production of B mesons, but only from electron-positron colliding beam machines in which all of the interesting processes happen inside the vacuum pipe. Heroic attempts have been made to deduce the lifetime of the B from these data but the results are not yet completely convincing.

The figure shows the convincing example of the production and decay of a pair of

beauty particles found in a nuclear emulsion stack that had been exposed to a 350 GeV p-minus beam at the CERN super proton-synchrotron by the WA75 collaboration (Brussels, Dublin, CERN, University College and Birkbeck College, London, Torino, Rome, Bari and Nagoya with associated Japanese laboratories). All five vertices are well resolved. The p-minus meson comes in from the left on the sketch and strikes a nucleus in the emulsion. Among the tracks coming from that primary interaction vertex, the B⁻ travels about half a millimetre in the emulsion before it decays to a muon (μ^-). Just over half a millimetre from the B⁻ decay-vertex there is a 'four-prong' vertex that is taken to be the decay point of a neutral charmed particle, a D⁰ meson. Another neutral decay is seen some millimetres from the primary vertex. This is taken to



be the decay point of an anti- B^0 meson (B^0) that produces a negative charmed particle, probably a D^- , which itself decays after some millimetres, giving rise to another muon.

The process illustrated in the figure seems very simple, yet scanners using high-powered microscopes took months to put the event together, even after the primary vertex had been located. The 'needles' to be searched for are tracks made up of grains less than a micrometre across. The experiment used 80 litres of Fuji and Ilford emulsion — a 'haystack' of 8×10^7 cubic millimetres. The problem was made manageable both by surrounding the emulsion with electronic detectors which identified the candidate events that were likely to contain beauty particles and by pointing back the tracks from such events to define a search-volume of about a cubic millimetre for each candidate. To avoid overcrowding the emulsion, with lots of beam tracks in one place, it was moved periodically on a precision stage.

Many of the tracks seen in the emulsion correspond to those reconstructed from the high-resolution silicon-strip detectors that were used to predict the search volume. The muon tracks are especially important in identifying beauty candidates and for this event both of them were seen by the silicon detectors and in a downstream muon spectrometer where their momenta were measured after they had passed through a tungsten and steel wall that absorbed the other (strongly interacting) particles. The transverse momentum of the muon from the B^- was particularly large, consistent only with

beauty decay, too big to have come from the decay of a lighter charm particle. The presence of this muon, recognized by fast electronics 'on-line' within a few microseconds of the interaction taking place, gave the primary 'event trigger' that allowed all available information from the detectors to be written on magnetic tape. Analysis of the data off-line picked up the second muon from the D^- decay, which made the event a prime candidate to be notified to the emulsion scanners.

Is one event so important? Even with two beauty tracks it cannot properly measure the beauty lifetime. But the decay times estimated from it are significantly shorter than those from the electron-positron machines, a few times 10^{-13} seconds, as opposed to more than 10^{-12} seconds. The lifetime of beauty is one of the constraints on part of our standard model of the electroweak interaction. Theorists were becoming embarrassed by long beauty lifetimes. The parameters of the model (the elements of the Kobayashi-Maskawa matrix) were becoming strained beyond reasonable limits. The WA75 event suggests that, once again, experimental results may be reinforcing the simplest possible model for weak processes. The group expect eventually to find enough needles in their haystack to measure the lifetime properly. For now, it is good to know for sure that beauty particles exist. $\square$

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Ocean Drilling Program

Looking down an old hole

from the Leg 102 shipboard scientific party*

ONE of the problems of drilling is that it is virtually impossible to extract a complete core from a drill-hole and one can never be certain just what is missing. Fortunately there are a myriad of techniques available to make *in situ* geophysical measurements of the rocks exposed in the sides of a hole. Because such measurements had been so successful in characterizing young (6.2 Myr) oceanic crust at Site 504 in the Pacific, it was decided that Leg 102 should re-investigate Hole 418A on the southern edge of the Bermuda Rise (25° 02'N; 68° 04'W), from which an incomplete core was taken eight years ago as part of the Deep Sea Drilling Project. The geophysical data obtained from these 110-Myr-old oceanic basalts show some interesting differences compared with the young Pacific crust.

Undeterred by the prospect of finding a hole only 25 cm across in water over 5 km deep, the new Ocean Drilling Program's drillship *JOIDES Resolution* arrived

at Site 418 on 21 March, 1985. For the next 2½ weeks borehole geophysical measurements were made to depths of 800 m below the sea floor in the upper 500 m of basalt. The results obtained can be interpreted in terms of ageing of the basaltic crust.

Acoustic velocity and waveform logs showed relatively high compressional and shear wave velocities (4.5–6.0 and 2.1–3.4 kms^{-1} respectively) throughout the upper 500 m of the oceanic crust (layer 2), with slow values occurring in the predominant weathered pillow basalt and breccia units and fast values in the massive basalts, which would be expected to be less prone to alteration. A similar difference was shown by induction and focused electrical resistivity logs, with resistivities of 40–200 ohm-m through the pillow basalts and breccias and values greater than 1000 ohm-m in the massive units. The upper 500 m below sea floor were distinguished by unusually high gamma ray activ-

ity and relatively high formation porosities, as determined by density and neutron porosity logs. The base of this section was marked by a sharp magnetic polarity reversal, followed by a notable low on the magnetic susceptibility log from 500 to 600 m below sea floor, then a gradual increase to the bottom of the log. Unusually for holes drilled in Layer 2, caliper logs demonstrated that the hole was 'to gauge' throughout. But perhaps most excitingly, temperature measurements indicated conductive, rather than convective, heat flow in the old basalt. This suggests a less active hydrothermal regime, a view supported by the water chemistry, which showed a difference between water within the borehole and that in the surrounding rocks, the borehole water being enriched in Ca^{2+} and depleted in Mg^{2+} and K^+ .

Our preliminary interpretation of the borehole geophysical and geochemical data is that the old crust at this site has been sealed by alteration products (clays and carbonates) within the pillow basalt units, and between layers of pillow and massive basalt, restricting the movement of pore water and causing geophysical Layer 2A now to be absent. This is in contrast to the interpretation of similar studies at Hole 504B in young crust in the Pacific. At that site, Layer 2A is present, the upper basalt is relatively porous and permeable, and observed underpressures and flow of seawater into the upper permeable zone suggest weak hydrothermal convection within the pillow lavas.

A problem facing all investigations using drill-holes is to extrapolate from the downhole log to the regional geology. Large-scale seismic experiments may be used to estimate large-scale fracturing and porosity and to determine the velocity structure, anisotropy and heterogeneity of the crust. Such seismic work may also indicate the presence of deep reflectors, for example, the boundary between extrusive and intrusive basalts within the oceanic crust. To meet these objectives, an oblique ship-to-ship seismic experiment was conducted at Site 418 using the RV *Fred H. Moore* as the shooting ship and the *JOIDES Resolution* as the receiving vessel. Shot points were located along radii and concentric circles centred on the borehole. A close-spaced seismic reflection survey was made to obtain basement topographical corrections. Seismic anisotropy and lateral heterogeneity were clearly established. Because of the techniques and equipment used, the data are of high quality, making it possible to extract evidence of deep reflectors, either within the crust, if they exist, or at the Moho. $\square$

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What was all the fuss about?

Richard Dawkins

Time Frames: The Rethinking of Darwinian Evolution and the Theory of Punctuated Equilibria.

By Niles Eldredge.

Simon & Schuster: 1985. Pp.240. \$16.95. To be published in Britain by Heinemann in January 1986.

THE alert reader of scientific magazines can hardly fail to be aware of a widespread lay rumour of something rotten in the state of Darwinism. We have seen a spate of more or less silly books by fundamentalist crackpots, cosmologists, biochemists and BBC producers, all banging the same drum. Two quotations from reviews give the general flavour: "... a highly readable non-technical account of just why Darwinism has come to be seen as a totally inadequate account of how life came to evolve on this planet"; "An original challenge to an increasingly beleaguered orthodoxy". There is nothing original in the objections deployed by these books, just the old favourites ("The eye is too complicated to have evolved by blind 'chance' ", and the rest) that Darwin himself raised — and demolished. These arguments have been repeatedly rediscovered, and the answers to them repeatedly ignored or misunderstood, during each of the decades since Darwin first discussed them. So why have gleeful rumours of a "beleaguered orthodoxy" spread particularly in the latest decade? Has there been some fresh impetus, some source of new encouragement for the old fundamentalists and fellow travellers?

Like any vigorously flourishing field of science, Darwinism has always had its in-house disputes. If these had been noticed by a wider public, it is likely that they would have been seized upon as evidence that the Darwinian "orthodoxy" was crumbling, so ready is the motivation to find such evidence. The effect is heightened if any chink of encouragement is offered by professionals on the inside of the business. I regret to say that more than a chink was offered by champions of "punctuated equilibria". The debate about this interesting little theory is a technical, parochial affair if ever there was one. It lies firmly *within* the neo-Darwinian synthesis. It is no more revolutionary than many another argument that has enriched the synthetic theory, for instance that over the "gene as the unit of selection". Yet punctuationism is widely thought to be revolutionary and antithetical to neo-Darwinism, for the simple reason that its chief advocates have said that it is; said so, moreover, in loud and eloquent voices, making frequent and skilful use of the mass media. The theory, in short, stands out from other glosses on the neo-Darwinian synthesis in one re-

spect only: it has enjoyed brilliant public relations and stage management.

Dr Eldredge is one of the two originators of the theory of punctuated equilibria. He is neither a cosmologist nor a biochemist, but a genuine expert on evolution. He has to be taken seriously, and he offers us here a pleasantly written account of the punctuation theory. The book is partisan, and none the worse for that. It is engagingly personal: one has a glimpse of what it is like to be a good palaeontologist and to love trilobites. It lapses into skipworthiness only when Eldredge indulges his predilection for woolly analogies with the social sciences, and his curious enthusiasm for someone called Frederick Teggart.

There is a useful reprinting of the original paper by Eldredge and Gould which launched the theory of punctuated equilibria in 1972. Re-reading this paper, and reading the new book, I was struck by the comparative modesty of both. The more self-aggrandizing hype seems to have been a phenomenon of the intervening years, climaxing, perhaps, in 1980 when Stephen Gould pronounced the

synthetic theory "effectively dead", and when a science journalist, sycophantic enough to deserve the title Gould's Poodle, hailed "An historic conference in Chicago" which "challenges the four-decade long dominance of the Modern Synthesis". If the book signals the end of that grandiloquent era it is very welcome, for the misunderstood rhetoric of middle-period punctuationism gave abundant aid and comfort to creationists and other enemies of scientific truth, and may well have been largely responsible for the ill-informed rumour that I mentioned at the outset. It is only fair to add that both Eldredge and Gould have been latter-day bulldogs in the fight against American fundamentalism.

Darwin's own bulldog, Huxley, as Eldredge reminds us yet again, warned him against his insistent gradualism, but Darwin had good reason. His theory was largely aimed at replacing creationism as an explanation of how living complexity could arise out of simplicity. Complexity cannot spring up in a single stroke of chance: that would be like hitting upon the combination number that opens a bank vault. But a whole series of tiny chance steps, if non-randomly selected, can build up almost limitless complexity of adaptation. It is as though the vault's door were to open another chink every time the number on the dials moved a little closer to the winning number. Gradualness is of the essence. In the context of the fight against creationism, gradualism is more or less synonymous with evolution itself. If you throw out gradualness you throw out the very thing that makes evolution more

IMAGE
UNAVAILABLE
FOR
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REASONS

The Chinese way of bookselling — detail of a bookshop of Northern Sung appearing in a long scroll painting, Spring Festival on the River (early twelfth century AD). The illustration is taken from Part I, Paper and Printing, by Tsien Tsuen-Hsueh, of Vol. 5 of Joseph Needham's Science and Civilisation in China. Publisher is Cambridge University Press, price £45, \$89.50.

plausible than creation. Creation is a special case of saltation — the *saltus* is the large jump from nothing to fully formed modern life. When you think of what Darwin was fighting against, is it any wonder that he continually returned to the theme of slow, gradual, step-by-step change?

Punctuationists are in favour of a form of jumpy evolution, but it is leagues away from the saltationism that Darwin was fighting. The punctuationists' "real gaps" in the fossil record are gaps only if you confine your digging to one place. They are all too easily confused with the sort of gaps that only a divine architect could bridge. In Darwin's sense of the term, Eldredge and his colleagues must be gradualists — they would have to be creationists if they weren't. But they believe that the episodes of step-by-step, gradual evolutionary change are sporadic, separated in space and therefore hard to find in the fossils, and separated in time by long periods of stagnation. All that the punctuationists have really done is to inject periods of stagnation into an otherwise traditional Darwinian picture of gradual evolution with allopatric speciation. Whether they are right to do so is an empirical matter that palaeontologists continue to debate. The upshot of this factual debate, whichever way it goes in particular cases, will upset nobody, and certainly not "the four-decade long dominance of the Modern Synthesis". Darwin himself would have been equally happy either way, because evolutionary change is still gradual *during the times that it is actually happening*. The punctuationists deluded themselves that they were saying something revolutionary. And it was all due to a simple verbal misunderstanding: a confusion of two senses of the word "gradual".

There is a second controversy which does, in a sense, move outside the neo-Darwinian synthesis, narrowly interpreted. This is about whether a form of natural selection operates at the level of entire lineages, as well as at the level of individual reproduction stressed by Darwin and neo-Darwinism. Eldredge is particularly involved with this now and he makes some worthwhile points, especially about the "effect hypothesis", and about the unitary status of the species as a player in the evolutionary game. But he agrees that species-level selection can't explain the evolution of adaptations: eyes, ears, knee joints, spider webs, behaviour patterns, everything, in short, that many of us want a theory of evolution to explain. Species selection may happen, but it doesn't seem to *do* anything much. After reading this book, one is left with a feeling of "What was all the fuss about?", and a suspicion that Eldredge may be left with it too. □

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Unseen horizons: the future of astronomy

George Efstathiou

The Invisible Universe: Probing the Frontiers of Astrophysics. By George B. Field and Eric J. Chaisson. *Birkhäuser*: 1985. Pp.195. SwFr. 58, \$19.95.

THE unveiling of the invisible Universe represents the most significant development in astronomy of the twentieth century. Since Galileo's time, astronomers have observed the sky with optical telescopes using visible light. Professors Field and Chaisson focus on the rest of the electromagnetic spectrum — the invisible radiation which holds the clue to many of the most spectacular phenomena in our Universe.

Their account is based loosely on a major report, *Astronomy and Astrophysics for the 1980s*, which was compiled for the United States Federal Government by a committee chaired by Professor Field. In the book Field and Chaisson provide a popular account of the advances we can expect from the next generation of satellites and telescopes. The choice of subject matter is wide-ranging — from Solar Sys-

tem astronomy to high-energy particle physics — and with the strikingly beautiful colour pictures cannot fail to excite the reader's imagination. The discussion is lucid, informed and supported by a useful glossary, although in places (for example the section on interstellar molecules) it does become quite technical and probably beyond the understanding of most lay readers. Throughout the book, the glittering array of new facilities provides the central theme but, curiously, the authors do not distinguish between those that are approved, such as the Space Telescope, and those that may never be built.

In some places, the discussion is too brief to be very helpful. For example, the Field committee concluded that an explanation for the vast amounts of energy produced by quasars is one of the most important of the current problems in astronomy, and so it was disappointing to find that only a few pages are devoted to the puzzle. Nevertheless, I enjoyed this book. Anybody who wants an authoritative account of future directions in astronomy would do well to read it, and it will be especially useful to undergraduates who are thinking of doing research. □

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Coming devices

C.R. Pidgeon

Free-Electron Lasers. By Thomas C. Marshall. *Macmillan, New York/Collier Macmillan, London*: 1985. Pp.191. \$24.95, £29.95.

Two years ago one could say that although many experiments had demonstrated the free-electron laser (FEL) as an amplifier of laser light, the gain threshold for actual laser oscillation in a mirrored optical cavity had only twice been surpassed. A renewed burst of activity since then has resulted in demonstrations of FEL oscillation in many countries, and at wavelengths all the way from the millimeter to the visible region of the spectrum. At the most recent meeting of the regular series of FEL conferences (Rome, 1984) it was predicted that in the near future ultraviolet, vacuum ultraviolet and soft X-radiation will be generated by FEL devices. As Thomas C. Marshall states in the present work, the FEL will be increasingly leaving the designer's laboratory and becoming available for research.

In a FEL amplifier one observes or infers the amplification of an external laser beam as it interacts with an undulating electron beam passing through the periodic magnetostatic structure of a "wiggler". By contrast, an FEL oscillator

produces a laser beam without the need for an external laser. The spontaneous radiation generated by undulating electron bunches in the wiggler is reflected back and forth between a pair of end mirrors, generating enough stimulated emission in successive electron bunches to produce and sustain a laser beam.

Marshall has provided a worthwhile treatment of the operation and characteristics of the FEL, with a useful historical and conceptual review, and bibliography. Most aspects of the subject are covered, including both single-particle (or Compton) systems and collective-particle (or Raman) systems. The author has attempted — reasonably successfully, I think — to write for the larger scientific community something between "news" and a professional review paper. The book will also be useful to the FEL community itself as a less-specialized source of information in what has by now become quite a broad and fast-moving field, though the most detailed and up-to-date information must of course still be obtained from the proceedings of the yearly workshops held on FEL physics and devices. The only slight quibble I have, perhaps inevitable for a book of this length, is that in several places quite complex results are merely quoted, without derivation or even comment from other sources. □

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Thinking about the ways of science

John Ziman

Popper Selections.* Edited by David Miller. Princeton University Press: 1985. Pp.479. Hbk \$32.50; pbk \$9.95.

ANY natural scientist interested in the philosophy of science will be acquainted with the work of Karl Popper. His identification of "falsifiability" as the hallmark of a scientific theory is now so widely accepted that scientists even use it as a weapon in purely scientific controversies: "I know my theory is conjectural, but at least it could be refuted by the following experiment, whereas yours is so broad and vague that it could not be disproved" etc., etc.

Many scientists attracted to Popper's ideas will have read their way with profit and pleasure through his half-dozen or so major books, and come to appreciate the breadth and depth of his thought on the meaning and social significance of the scientific approach to the world — otherwise one would have had to be content with a version of these ideas at second hand, in one of the books that have been written about them by other philosophers. But Popper is not one of those gurus whose jewels can only be appreciated by cleaning away a mass of obscuring verbiage. Not all his writing is perfectly clear and transparent at the first reading — he is dealing, after all, with very difficult questions — but there are many passages that sum up his arguments with extreme clarity and simplicity. David Miller's selection of some of these passages is thus an invaluable introduction to the work of one of the leading thinkers of our times.

It is not difficult to understand why working scientists are drawn to Popper's philosophy. His characterization of the work of research as the evolution of a body of knowledge through a process of trial and error resonates with their own experience, both as would-be discoverers and as critics of discovery claims. This overall account of the scientific process has, of course, been attacked in detail by numerous other philosophers, but it still stands firm. Indeed, the most striking feature of Popper's thinking and writing is that it is so *robust*. He fastens on a few very solid principles, and stands firmly on them throughout — the corrigibility of all scientific knowledge, the logical force of a disconfirmed prediction, the vapidness of philosophical idealism and, in the social sciences, the absurdity of the assumption that people always behave rationally. Knock-me-down arguments are not welcomed in professional academic life, which thrives on subtlety and equivoca-

tion, but I really wonder how the modern exponents of the sociology of knowledge would answer Popper's devastation of their position, originally published 40 years ago and still entirely valid.

Of course there are situations where reliance on a few apparently sound general principles can be misleading. Popper was once led into asserting that the principle of biological evolution was not a "scientific" hypothesis because it seemed essentially tautological: biologists will be pleased to read here a passage written in 1977 where he gracefully acknowledges his error on this point. I suspect that his remarks on "The Mind-Body Problem" and "The Self" will not stand for long, as natural,



Man of principles — Karl Popper, a portrait from around 1950.

and social and behavioural scientists collaborate more closely in their accounts of consciousness and cognition. It is also surprising that the very clear conception of the "public" character of the scientific process, through the intersubjectivity of observation and the mutuality of creative criticism, which he gave in a page or two in 1945, scarcely figures in his later work. Again, his notion of "world 3" — that is, the domain of "the objective contents of thought", such as scientific theories and works of art — could have been made much more convincing and fruitful if it had been firmly related to the function of symbolic communication in human societies.

But in the end one comes back to Popper for his incisive commentary on the *strategies* of scientific and political action. He does not provide us with *rules* on which to act — indeed, his first rule would be that there are no such rules — but his critique of scientism and historicism is founded upon *precepts* which every scientist, every citizen, would ignore at their peril. □

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Speechless in Java

John C. Marshall

Ancestral Voices: Language and the Evolution of Human Consciousness. By Curtis G. Smith. Prentice-Hall: 1985. Pp.178. Hbk \$14.95, £17.50; pbk \$8.95, £9.60.

THE "ancestral voices" that Kubla Khan heard in Xanadu were "prophesying war"; the voices that appear in Curtis Smith's title are those of our purported evolutionary precursors who first developed the capacity for symbolic communication. But the parallels are somewhat too close for comfort. The archaeological record, Smith conjectures, suggests that linguistically-skilled Cro-Magnon "with his superior culture and technology" exterminated speechless Neanderthal man, "his closest rival for the riches of Europe".

Like so many eminent scholars before him, Curtis Smith, a professor of biological sciences at Mount Holyoke College, Massachusetts, has failed to resist the temptation to speculate on the evolution of language and consciousness. Professor Smith's mixture is fairly conventional: fossil remains, flint tools and progressive enlargement of the cerebrum, combined with attempts to teach chimpanzees American Sign Language and to communicate with hypothetical intelligences on other planets. The problem, however, is that whilst we have undoubtedly learnt much about the structure of the brain and much about the structure of human language we still have few (or no) coherent ideas about how to relate the two. That localized damage to different parts of the brain can result in differential loss of aspects of linguistic capacity and skill places constraints upon speculation but leaves the central issue untouched. Professor Smith has no more insight into that issue — how the brains of human beings differ from those of other higher primates such that only human brains can support a syntactically-structured language — than do the rest of us.

This leaves Smith to make some none-too-accurate general remarks about the functional organization of the brain. Thus he asserts that the brain resembles "an enormous hologram" in that "all of the neural symbols used for language, visual memories, music, feelings, are stored 'everywhere' ". It may turn out that this claim is true, but it is grossly misleading to present the holographic hypothesis as already established fact; and it is even worse to do so without giving any indication of how such distributed information-storage could be compatible with the specificity of deficit that is so often observed after focal damage. Smith's convictions are rarely backed by evidence. He simply states, for example, that "amnesias are defects in the retrieval system, not actual

*In Britain published by Fontana as *A Pocket Popper*, pbk £4.95.

loss of the memories themselves" without indicating that such matters are still under active debate. He is likewise convinced that the storage and recognition of faces is a holographic process, but does not bother to mention that in (monkey) cortex there do appear to be single cells tuned to particular familiar faces. His interpretation of commissurotomy ("split-brain") patients also prejudges the outcome of current discussion. *Contra* Smith's confident assertions, there are reputable scientists who do believe that we have two brains (hemispheres) and that (in the vast majority of people) language capacity and skill is limited to one of them.

Ancestral Voices is clearly intended as a popular, "lay" book on a topic that very naturally fascinates many people well beyond the confines of the neuroscience community. All the more important, then, that a fair picture should be painted

of the limits of current knowledge, and of the disagreements between those who work in the area. Professor Smith's efforts do not meet these requirements. Whitney's trenchant summary (1870) of theories of the origin of language comes to mind all too often:

No theme in linguistic science is more often and more voluminously treated than this, and by scholars of every grade and tendency; nor any, it may be added, with less profitable result in proportion to the labor expended.

If, despite Whitney's pessimism, the urge to read a book on the topic remains, I would recommend that one searched for a copy of George John Romanes' Victorian masterpiece, *Mental Evolution in Man* (1888). □

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Temporal questions

James A. Secord

The Dark Abyss of Time: The History of the Earth and the History of Nations from Hooke to Vico. By Paolo Rossi. Translated by Lydia G. Cochrane. University of Chicago Press: 1985. Pp.338. £32.25, \$35.

ANYONE familiar with the development of science in the past few decades will recognize that disciplinary boundaries are far from static. Sciences move closer to and further away from one another; they break up, disappear or regroup into new subjects and specialities. Move back 300 years, and the changes involve not only science but the whole realm of human knowledge. What, for example, do fossil ammonites have in common with Chinese writing and the Egyptian pyramids? To modern ears the question seems absurd, for these subjects now belong in very distinctive scientific specialities, and any relation between them would seem a matter of chance. But in the seventeenth century their affinity was no riddle, but a feature of intellectual life that was taken for granted. Fossils, ancient languages and relics of lost civilizations all had a place in a unified study of the past: they were "signs of time". Hence the original Italian title of Paolo Rossi's important book *I Segni del Tempo*, now available to a wider audience in an English translation.

The objects grouped together here were also "signs of time" in another sense, as indications that the world might be far older than was generally thought. It is difficult for readers steeped in the time-scale of modern geology to recapture the threat that seemingly limitless time posed to men of science in the late seventeenth and early eighteenth centuries. The Copernican revolution and its consequences are familiar

from many historical discussions. But as Rossi notes, "there has been no such insistence, in the same way and with the same intensity, on other, no less decisive changes" — notably our perception of time. In the late seventeenth century, the direct and best testimony on the early ages of the world's history — the Bible — rendered an estimate of some few thousand years since the beginning not only intellectually respectable, but almost inescapable. By the end of the eighteenth, scholars looked down into what Buffon called "the dark abyss of time", and in advanced circles an age for the Earth of millions of years had become a distinct possibility. The Bible was being replaced as a secure source of knowledge by broken fragments of strata, old legends and cryptic languages. The old authority had provided at least the possibility of a single coherent narrative for the history of the world; the new ones could only be deciphered piecemeal and with difficulty.

As Rossi demonstrates, this change held major consequences for the organization of knowledge. In Genesis, the world commenced in the space of a few days. This meant that the history of the Earth and the history of man could be studied as one subject, for the two histories were almost precisely coextensive. This is a basic point, but one all too often set aside by those concerned with finding the roots of modern scientific disciplines. For instance, Robert Hooke, John Woodward, William Whiston and Thomas Burnet are familiar names in the history of geology. Rossi shows that their writings need to be read alongside the works of less familiar authors such as William Warburton, Isaac de Lapeyrière and Martino Martini. Some of these men are well known in accounts of the origins of other disciplines (Egyptology, for example), while some have fallen into obscurity. By rescuing them from oblivion and reconstructing the character of their original concerns, Rossi traces the

contours of a fascinating and alien map of knowledge.

The Dark Abyss of Time looks particularly closely at those who maintained the authority of scripture against Hobbists, atheists, sceptics, pre-Adamites and others ready to use new empirical findings to undermine religious certainty and the social fabric. Discussion of these "defence mechanisms" is divided into three parts, devoted respectively to Earth history, human history, and barbarism and language.

Rossi is especially concerned to show how each of these topics became entwined in the making of a single major text in early modern intellectual history, Giambattista Vico's *Nuova Scienza*, published in its final form in 1744. Usually hailed as a founding figure of historicism and a precursor of Benedetto Croce, Vico appears here in dialogue with contemporary figures and themes. With scholarly finesse, Rossi depicts the author of the *Nuovo Scienza* picking his way "across the scorching hot, difficult terrain" of controversies about the origin of man and the Earth. Far from being an isolated forerunner, Vico can be seen charting a new path through territory that had been an intellectual battleground for decades.

This is an impressively researched book, but one with very few concessions to students or non-specialist readers. (A good introduction is available in the first two chapters of Martin J. S. Rudwick's *Meaning of Fossils*, recently reissued in paperback.) Some of the difficulties are simply a matter of style, especially as the analysis has a tendency to lose momentum in lengthy summaries of cosmological theories. Individual chapters are not clearly connected, and have something of the character of separate essays. Moreover, the main thrust of the argument is occasionally obscured by traces of the forward-looking disciplinary history that most of the book rightly questions. Chapters on Kelvin and the age of the Earth problem (and indeed, those on Buffon and James Hutton) seem especially out of place. The preface makes the real point of the book clear.

While placing Vico in a fresh perspective, Rossi has made a major foray into a vast and unexplored territory in the literature of early modern science, pointing a way that others will want to follow. At first glance, his book may seem to deal with a variety of subjects, many not obviously relevant to the history of science. As a result, there is a real danger that readers concerned with the development of a single discipline will turn only to those chapters that immediately interest them. Resist the temptation, for such an approach perpetuates precisely those intellectual blinkers that so frequently limit an appreciation of other ways of seeing the world. □

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Oblateness of the Sun in 1983 and relativity

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Measurements of the solar oblateness obtained during 1983 from Mount Wilson, California, yield a value with an upper bound only half of that observed in 1966. This difference may support the conjecture that the solar quadrupole moment slowly oscillates. A knowledge of the character of such an oscillation, if it occurs, would be needed to test Einstein's relativity theory using Mercury's orbital motion.

THE motion of the perihelion of Mercury's orbit provided the first and potentially one of the most important tests of Einstein's general relativity theory, but because of uncertainty concerning the solar quadrupole moment, this test has long been in doubt. In 1966, a measurement of the solar oblateness^{1,2} gave a value³ of $\Delta r = r_{\text{eq}} - r_{\text{p}} = 41.9 \pm 3.3$ arc ms (or 30.6 ± 2.4 km) for the excess equatorial radius of the Sun, that is the fractional equatorial excess of $\Delta r/r = 4.34 \times 10^{-5} \pm 0.34 \times 10^{-5}$. This seemed to imply a solar quadrupole moment large enough to cause a significant rotation of Mercury's perihelion.

This oblateness was first interpreted¹ as the effect of a rapidly rotating solar core, but the indication that the distortion was rotating rigidly across the Sun's surface with a 12.38 ± 0.10 day sidereal period^{3,4} cast doubt on this interpretation. It seemed more likely that the distortion resulted from a strong magnetic field, primarily toroidal, in the solar core rotating with this period⁵⁻⁷. Such an explanation could permit long-period toroidal oscillations of the core. It has been speculated that the sunspot cycle might result from a magnetic field generated by such an oscillation⁶. If so, the solar oblateness might oscillate, varying with the sunspot cycle. This test of general relativity must take such a variation into account, if it occurs. There is evidence that the solar cycle is associated with a precisely tuned oscillator deep in the Sun^{6,8}.

In 1975 a substantially smaller solar oblateness was reported⁹ and Hill *et al.*¹⁰ have suggested recently that the spectrum of solar oscillations they have found implies a rapidly rotating solar core and a significant quadrupole moment.

More recently, observations of the 5-min oscillations have given a core rotation rate roughly twice that of the solar surface, a core rotation which is too slow to yield a quadrupole moment significant enough for the relativity test¹¹.

1983 observations

During 10 April to 27 September 1983, measurements of the Sun's shape were obtained (~ 8 h per day) by one of us (K.G.L.) on Mount Wilson, California, with an improved version of the Princeton solar oblateness telescope which had been used in 1966 to measure the oblateness of the Sun². As in the earlier version of the telescope, the Sun's image is projected on one of three occulting disks of different diameter, exposing 9–20 arc s at the Sun's limb. This exposed limb is scanned at ~ 10 Hz using a rotating wheel containing two diametrically opposed 2° apertures to look for the variation in the light flux with angular position. An ellipticity of the solar disk would cause a second harmonic variation of the light flux with angular position on the limb. But at least part of such a variation could result from a 'brightness signal', a variation of the brightness of the solar disk with angular position.

Mount Wilson is better than the Princeton site in one important aspect: it yields a much greater fraction of days with a clear

sky. But in another aspect it seems to be less satisfactory: at the Princeton site the flat grassy terrain apparently resulted in a symmetry in the locally induced atmospheric distortion of the Sun that simplified the data analysis, this symmetry was not present in the Mount Wilson data, probably because of the mountainous terrain.

The principal improvements made to the instrument are (1) in the modified instrument, the light flux from the partially occulted Sun is averaged in 128 channels 1.41° wide (in 1966, only the second harmonic sine and cosine distributions of the light flux were measured). (2) The servo system centering the Sun's image on the occulting disk is based on a new, significantly improved, technique. It includes both fast and slow (integrating) servo loops. (3) The two diametrically opposed scanning apertures are the same size. This eliminates from the total light flux, in first order, signals induced by small solar-image displacements. (4) Measurements are made at eight angular positions of the vertically mounted telescope, eliminating telescope errors up to the 16th harmonic. (5) The measurements are made simultaneously in two wide colour bands centred at 'green', $0.53 \mu\text{m}$, and at 'red', $0.80 \mu\text{m}$. These bands are, respectively, 0.08 and $0.30 \mu\text{m}$ wide.

The light flux measured at 128 angular positions permits the direct detection and rejection of channels containing the signals from substantial facular patches. A channel is rejected (for the whole day and for all three disks) if the daily mean of the excess signal in the channel exceeds 3σ . (In 1966, corrections for faculae were made indirectly, by least squares, assuming that the facular signal was proportional to the area of a facular patch.)

Using the 1983 data, the new instrument has permitted a direct determination of the facular contrast near the solar limb by Libbrecht and Kuhn¹². The variation of the facular signal with the amount of exposed limb is consistent with the 1966 results¹³. Evidence for a fourth harmonic variation of the effective temperature about the solar limb with an amplitude of $0.6^\circ \pm 0.1^\circ$ has been found in the 1983 data by Kuhn *et al.*¹⁴.

As in 1966, the second harmonic corrections for astigmatic mirror errors are made by combining data obtained with the mirrors in four different combinations of positions, the primary and secondary mirrors each being in two positions 90° apart, and then including the four astigmatic mirror parameters in the least-square fits to the data.

Geometrical optics provide an inadequate description of the mirror-induced distortion of the Sun's image: for the distortion parameters obtained from the least-square fits depend sensitively on both the colour and the amount of exposed limb. It seems likely that the mirror distortion is a diffraction effect resulting from $\lambda/20$ roughness of the mirror surfaces on a scale of a few centimetres.

After the above correction is made, a small residual error remains in the correction for the primary mirror. It is eliminated by assuming that the mirror distortion is a function of the angle of incidence of sunlight on the mirror and including this type of dependence in the least-squares fit.

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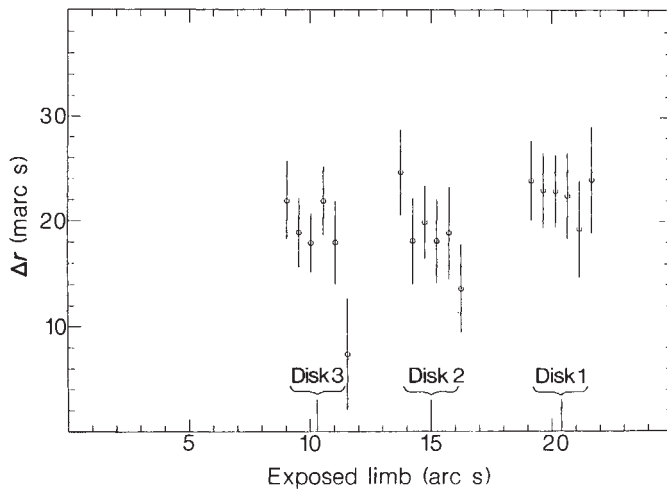


Fig. 1 Green solar oblateness. The values of Δr (in arc ms) derived from equation (3) are plotted for both colours and each solar-hour angle bin as a function of the exposed solar limb (in arc s).

After eliminating the channels contaminated by identifiable facular brightness signals, the second harmonic sine and cosine components of the light flux are obtained by least squares from the data, with the angular coordinate chosen to be zero at the terrestrial north point on the solar disk (not the solar pole). After dividing by the solar brightness at the edge of the occulting disk (and reversing the sign of the cosine component), twice the amplitudes of the sine and cosine components are expressed in arc ms and are designated Δr_d , the 'diagonal' component, and Δr_v , the 'vertical' component, of the solar ellipticity.

These two ellipticity components, corrected for mirror errors, are also corrected for the distortion induced by the refraction of a laminar atmosphere and are averaged for each day in six solar-hour angle bins, 20° wide, ranging from mean solar-hour angles of -50° to $+50^\circ$. In the discussion below, these six bins are designated $h = 1, 2, \dots, 6$, respectively.

The three occulting disks are designated $d = 1, 2$ and 3 and expose annuli at the solar limb with the seasonal mean widths for red of $\delta R = 18.1, 13.2$ and 9.2 arc s respectively and for green $\delta R = 20.4, 15.0$ and 10.3 arc s. These annular widths are defined by $\delta R = F/I$ where F is the light flux per degree past the disk and I is the solar brightness at the edge of the occulting disk expressed in flux units per deg arc s.

For an oblate Sun with oblateness $\Delta r = (r_{eq} - r_p)$, the solar oblateness contributes $\Delta r \sin 2P$ and $\Delta r \cos 2P$, respectively, to the diagonal and vertical components of the ellipticity. Here P is the counterclockwise position angle of the solar north pole relative to the north point on the solar disk and ranges from -25° to 25° during the observational period.

Atmospheric distortion

The distortion of the Sun's image by the Earth's atmosphere, assumed to be uniformly laminar, is not the only atmospheric distortion of the Sun. The temperature inhomogeneity of the air, primarily near the observatory, also distorts the Sun. This leads to the displacement of the time-averaged position of any stellar image and the spreading of the point image into a distorted seeing disk. We are concerned here with the time-averaged position and ellipticity of the seeing disk, the two dominant contributions to the elliptical distortion of the solar image having their origin in this local temperature inhomogeneity. They are called, respectively, the 'cylindrical lens' distortion and the 'anisotropic seeing' distortion². These are the two lowest order (dominant) terms in the distortion obtained from an expansion of the solar brightness, $I(x)$, in moments of the distorted seeing disk.

The cylindrical lens distortion is independent of the limb exposure, and hence of the disk number. The anisotropic seeing

Table 1 Results of least-squares fits, equation (3)

Disk	Bin	Δr	D	Δr	D
1	1	26.3	33.6	23.9	37.0
1	2	23.6	15.1	22.9	12.9
1	3	22.6	3.5	22.9	-0.8
1	4	22.5	-1.4	22.4	-4.3
1	5	18.8	-7.0	19.3	-7.2
1	6	27.0	-2.5	24.0	1.8
2	1	24.4	32.4	24.7	35.0
2	2	16.6	10.8	18.1	5.3
2	3	17.8	8.4	19.9	-0.1
2	4	17.6	3.7	18.1	-3.9
2	5	20.3	2.7	18.9	-1.7
2	6	17.6	3.0	13.6	1.3
3	1	27.1	23.8	21.9	33.4
3	2	19.8	7.6	18.9	10.3
3	3	13.5	2.0	17.9	1.6
3	4	17.2	4.4	21.9	1.5
3	5	16.2	9.7	18.0	3.0
3	6	12.9	19.4	7.4	12.3

distortion is proportional to the logarithmic radial derivative of the solar brightness at the edge of the occulting disk. For disks $d = 1, 2$ and 3 this is respectively for the red colour band:

$$S(d) = 1, 1.31 \text{ and } 1.84 \quad (1)$$

normalized to unity for disk number 1. For green the corresponding values are:

$$S(d) = 1, 1.28 \text{ and } 1.75 \quad (2)$$

respectively. These values are obtained from limb-darkening curves for these colours¹⁵.

For the vertical component, the atmospheric contribution to the apparent solar ellipticity is not statistically separable from the true solar oblateness, for $\cos 2P$ varies relatively little through the observing period. Another quite different difficulty with the vertical component of the data concerns the off-axis astigmatic error of the primary mirror. This cannot be determined from the mirror cycling, but it affects only the vertical component of the distortion. Because of these difficulties, the analysis discussed below is based only on the diagonal component of the ellipticity.

The contribution of the solar oblateness to the diagonal component of the solar ellipticity, $\Delta r \sin 2P$, can be determined by least squares using the known seasonal variation of $\sin 2P$. This determination of Δr can be made for each of the six solar-hour angle bins for each of the three disks and for each of the two colours by fitting

$$\Delta r \sin 2P + D \rightarrow \Delta r_d \quad (3)$$

Here $D(h, d)$ is interpreted as the seasonal mean of the local atmospheric contribution to Δr_d . To interpret Δr as the solar oblateness may be premature. There are two reasons for caution. First, there may be a fortuitous $\sin 2P$ seasonal variation in the atmospheric distortion of the Sun. Second, the excess light flux in the equatorial part of the Sun may be due, at least in part, to an excess solar brightness near the equator.

The results of the least-square fits, equation (3), to the diagonal component of the ellipticity are given in Table 1. The derived values of the Δr are plotted in Fig. 1 as a function of exposed limb for each bin. Here, and in Table 1, Δr and D are expressed in arc ms. The means of the standard errors of Δr are 3.6 and 4.0 arc ms for red and green, respectively. For D the mean errors are 1.9 and 2.0 arc ms respectively.

In the 36 fits, equation (3), 3σ deviations are much more numerous than the 6 expected with a normal distribution of errors. It is found that the 30 excess large deviations all occur in six scattered days. This strongly suggests that these 6 days

may be abnormal and that they should be eliminated from the original list of 131 days with data. With the criterion that there are 2 or more 3 σ deviations present, the 6 days with day numbers 98, 99, 129, 137, 159 and 175 are eliminated from the fit, equation (3), for the results given in Table 1 and in Fig. 1.

Note that the large variation of the atmospheric distortion D (as a function of h for fixed d) is not seen in Δr . Thus, it is unlikely that the atmospheric distortion has a fortuitous $\sin 2P$ dependence on time. The question of a 'brightness signal' contribution to the signal is discussed below.

For the hour angle bins $h = 1$ and 6, the Sun is fairly low in the sky and the atmospheric distortion is large. (Note the large values of $D(1, d)$ in Table 1.) The data in bins 1 and 6 may introduce both a systematic statistical bias and excess noise. In the analysis below, these bins will be dropped, but the analysis will also be carried out with these bins included.

We assume that the locally induced atmospheric distortion of the Sun, $D(h, d)$, consists of the sum of two parts, the cylindrical lens distortion, $C(h)$, and the anisotropic seeing distortion, $S(d)A(h)$, where $S(d)$ is given by equations (1) or (2). The least-square fit

$$\Delta r(d) \sin 2P + C(h) + S'(d)A(h) \rightarrow \Delta r_d \quad (4)$$

combines data from all three disks and is computed for each of the two colours. The oblateness $\Delta r(d)$ is a function of disk number to allow for the possibility of a contribution from a 'brightness signal'.

To test the atmospheric distortion model, $S'(d)$ is included with $C(h)$, $A(h)$ and Δr to be varied in the least-squares fits. The results obtained for the ratio $(S'(3)-1)/(S'(2)-1)$ are $2.77 \pm .35$ and 4.1 ± 1.1 respectively for the red and green signals and are in satisfactory agreement with the ratio 2.71 obtained from equations (1) and (2). Consequently, these equations are used when computing the corrections for atmospheric distortion, using equation (4).

The results obtained from equation (4) for red are $\Delta r(d) = 21.7 \pm 1.7$, 18.3 ± 1.7 and 16.6 ± 1.7 for $d = 1, 2$ and 3, and for green, 22.0 ± 1.9 , 18.8 ± 1.9 and 19.2 ± 1.9 arc ms. Note that, independently of the colour and the disk number, the six values of Δr do not differ significantly from their mean. For $C(h)$ and $A(h)$ the results are given in Table 2. The corresponding mean

Table 2 Atmospheric distortion (arc ms)

	h	2	3	4	5
Red	$C(h)$	23.0 ± 4.1	8.8 ± 4.1	-6.3 ± 4.2	-24.9 ± 4.3
Red	$A(h)$	-8.6 ± 2.9	-3.0 ± 2.9	6.2 ± 3.0	19.3 ± 3.0
Green	$C(h)$	12.1 ± 4.9	-4.0 ± 4.9	-13.4 ± 5.0	-19.8 ± 5.1
Green	$A(h)$	-1.9 ± 3.5	3.2 ± 3.5	8.3 ± 3.6	13.2 ± 3.7

estimated post-fit errors in the data points are for red, 18.8 ms, and for green, 20.5. The number of data points (20° binned means) is 1,359.

The four (or six) bins of data for each colour and disk, corrected for local atmospheric distortion using the results in Table 2, are averaged to form sets of daily averages. Only days for which data exist for all three disks are kept, a total of 121 days for the daily means of bins $h = 2$ to 4.

Brightness signal

To compute the brightness signal contribution to the apparent solar oblateness, we assume that the green brightness signals are greater than the red by a factor λ , to be determined. For excess light flux resulting from faculae, we measure $\lambda = 1.6$ and for a small change in the photospheric temperature we expect a value of 1.5.

The daily mean values of the diagonal component of the solar ellipticity, corrected as above, are assumed to be the sum of two

parts, a shape signal S and a brightness signal λB where $\lambda = 1$ for red.

The mean value M and the semi-difference D of the green and red signals are:

$$M = S + 0.5(\lambda + 1)B \quad (5)$$

$$D = 0.5(\lambda - 1)B$$

The colour difference D provides a measure of the brightness signal for each of the three disks.

The red and green day-to-day fluctuations (noise) are mainly in the shape signals. The variances of M , computed from the residuals after removing the fitted ellipticity, are 9–30 times as great as those of D . The 3×3 covariance matrix of D apparently requires two separate brightness signals common to all three disks and a low noise level for disks 1 and 2. The brightness signal is strongest in D_1 (disk 1) which is adopted as one of the 2 signals. The second $D' = D_2 - 0.70 D_1$ is orthogonal to D_1 .

The least-squares fit

$$\Delta r \sin 2P + c_1 D_1 + c_2 D' \rightarrow M \quad (6)$$

is used to fit simultaneously for the oblateness and the two components of the brightness signal. The results are given (in arc ms) in Table 3. The last column is the estimated standard error of the daily means of the solar ellipticity, M .

Table 3 Least-squares fits for the solar oblateness and brightness

Disk	Δr	C_1	C_2	σ
1	20.9 ± 1.9	$2.80 \pm .38$	$1.49 \pm .78$	10.36
2	18.5 ± 2.0	$1.73 \pm .41$	$0.69 \pm .84$	11.16
3	18.3 ± 1.8	$1.20 \pm .36$	$0.22 \pm .75$	9.87

The first brightness term is statistically significant for all three disks, but it contributes very little to the apparent oblateness of the Sun. This brightness varies on a timescale of 1 day but is not apparently concentrated at the equator or pole.

In Table 3, the mean of the three values of Δr is 19.2 ± 1.4 . The deviations of the three values of Δr from the mean are not statistically significant and these deviations provide no evidence for the existence of some additional (unmodelled) brightness signal.

The factor λ in equation (5) can be evaluated as $\lambda = (c_1 + 1)/(c_1 - 1)$ from the first line of Table 3, neglecting D' . This gives the result $\lambda = 2.1 \pm 0.3$, (-0.2). If the same fit, equation (6), is made using the daily means of the solar ellipticity which include the bins 1 and 6, the results for Δr are: 23.7 ± 1.8 , 19.2 ± 1.8 and 18.7 ± 1.8 ms.

The above formal standard deviation of the mean solar oblateness, 19.2 ± 1.4 arc ms, is not a realistic measure of the error. There could be a seasonal variation in the atmospheric distortion that would add to or subtract from the apparent oblateness, perhaps 1–3 arc ms. A much larger variation seems to be precluded by the absence of a marked $\sin 2P$ variation in the various weather parameters and in the declination of the Sun.

A more serious problem is the possible existence of another, colour-independent, brightness signal, for which $\lambda = 1$. Such a signal would escape the above analysis. There is no evidence for the existence of such a signal, for the oblateness is seen to be substantially independent of disk number. Nonetheless, a small signal of this type could exist and increase the error of the oblateness determination.

To include the effect of such a signal, we use the least-squares fit

$$\Delta r \sin 2P + BE(d) + c_1(d)D_1 + c_2(d)D' \rightarrow M \quad (7)$$

Here the second term is the new brightness signal and the third and fourth terms are those previously discussed. We assume that the colour-independent brightness signal is proportional to the exposed limb and this factor is $E(d) = 1, 0.73$ and 0.50 . The fit combines the data for all three values of the disk number d .

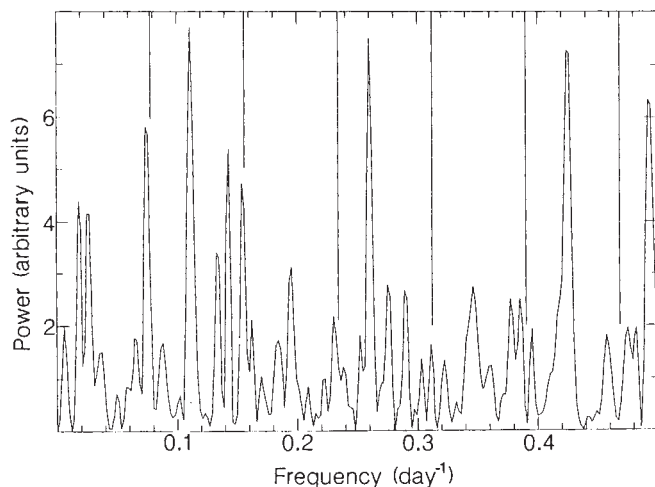


Fig. 2 The power spectrum of the residuals of M_3 (disk 3), obtained by removing the fitted value of $\Delta r \sin 2P$, from M . The vertical lines give the frequencies of the first six harmonics of the 1966 frequency, 0.07804 per day.

It gives the results $\Delta r = 15.4 \pm 4.1$ and $B = 5.2 \pm 5.3$ arc ms. Note that the brightness signal B is not statistically significant and that the change in the oblateness is only 1 standard deviation (s.d.). Nonetheless, the inclusion of this additional brightness term increases the error of the oblateness substantially, and the oblateness could be low enough to make the solar quadrupole moment negligibly small. If the daily means of the solar ellipticity which include the bins $h=1$ and 6 are used, the results $\Delta r = 13.0 \pm 3.9$ and $B = 10.2 \pm 5.0$ are obtained.

12.38-day rotational period

As noted above, the 1966 observations of the solar ellipticity were best interpreted as the effect of a solar distortion rotating rigidly over the surface with a (sidereal) period of 12.38 days. The first three harmonics of the synodic frequency, 0.07804 ± 0.0006 per day, appeared in the power spectrum of the residuals (after subtracting the fitted oblateness term). This periodic signal is much weaker in the 1983 data, if it is present at all.

The power spectrum of the residuals of M_3 (disk 3) is shown in Fig. 2. The vertical lines give the frequencies of the first six harmonics of the 1966 frequency, 0.07804. The first two harmonics may be present in the 1983 data but these two frequencies are not the two most prominent peaks in the power spectrum. The first harmonic is also present in M for the other two disks, where the noise level is higher, and in D_1 where the amplitude is 1.0 ms, but the error is only 0.3 ms.

A least-squares fit to the above two peaks for a rigidly rotating distortion yields a rotational frequency $f = 0.0792 \pm 0.0005$ per day, in good agreement with the frequency obtained from the 1966 data³. However, the amplitudes of the first two harmonics of the fitted function are only one-fifth and one-half of those obtained from the 1966 data.

In the power spectrum, there are four strong peaks for which we have no explanation. Could some of the other peaks be aliases of these four peaks (arising from the complexities of the window function due to missed observational days)? This is unlikely, for the aliases are very weak, with powers only a few per cent of the powers of the main peaks in the spectrum.

Despite the occurrence of the harmonics of $f = 0.0792 \text{ yr}^{-1}$ in the power spectrum, we do not believe that the existence of the 12.4-day rotational period in the data has been demonstrated. A much stronger case for the existence of this rotational signal was made using the 1966 data^{4,16}.

Conclusions

Assuming that the brightness signal is colour dependent, the value of the oblateness of the Sun obtained in 1983 is 19.2 ± 1.4 arc ms, where the error is only a formal s.d. A seasonal variation of the atmospheric distortion (in the form $\sin 2P$) might occur. It could have either sign and contribute an error to the oblateness determination, perhaps as great as ± 3 ms. If there should also be a colour-independent brightness signal proportional to the exposed limb (in addition to the colour-dependent one) the value 15.4 ± 4.1 would be obtained for the oblateness, where the error is again only formal. For both of these possibilities the 1983 result for the oblateness is $\ll 42 \pm 2$ ms obtained in 1966.

Unless there is some support for the existence of a colour-independent brightness signal, it seems reasonable to adopt 19.2 ± 1.4 ms for the oblateness, with the proviso that a more believable error might be ± 4 ms. Of this oblateness, 7.8 ms results from surface rotation (a centrifugal distortion of surface layers)¹⁷. The remainder, 11.4 ms (or $\Delta r/r = 1.2 \times 10^{-5}$), is associated with a quadrupole moment $J_2 = (2/3)(\Delta r/r) = 7.8 \times 10^{-6}$ which is only one-third of the value obtained from the 1966 observations.

The 1983 results seem to be compatible with the conjecture that the Sun has an oscillating quadrupole moment, but an extended series of observations would be needed to confirm this conclusion. The implications of the solar oblateness for Einstein's theory of gravitation will be unclear until the solar quadrupole moment and its possible variation with time are measured. The implications of an oscillating solar core for the solar cycle, and for the generation of solar magnetic fields, could be profound.

The classical astronomical test of Einstein's theory derived from the excess rotation of the perihelion of Mercury's orbit requires a knowledge of J_2 and its variation with time. If variation of J_2 were periodic with the sunspot cycle, a 22-yr mean would be adequate. More recent planetary radar observations of Mercury's motion can use time averages on a decade timescale. A Mercury satellite orbiter could reduce this timescale markedly and permit an observation of the time variation of the perihelion rotation. If the time variation of J_2 were also known, this would permit an accurate test of this relativistic effect on a short timescale¹⁸.

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Amino-acid sequence of the catalytic subunit of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ deduced from a complementary DNA

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We have isolated and characterized a complementary DNA for the catalytic subunit of the sheep kidney sodium/potassium-dependent ATPase. The 1,016-amino-acid protein seems to have eight transmembrane domains. The apparent ouabain binding site is located at the extracellular junction of two transmembrane domains and is linked to the phosphorylation site by a 60-amino-acid conserved sequence that may be a major channel for energy transduction.

It is known that the sodium/potassium-dependent ATPase $[(\text{Na}^+ + \text{K}^+)\text{ATPase}]$ is an integral membrane protein that directly couples the hydrolysis of ATP to the vectorial transport of Na^+ and K^+ across the plasma membrane. This in turn produces the electrochemical gradient of Na and K which is the primary source of energy for the active transport of various nutrients¹, the action potentials of excitable tissues such as muscle and nerve² and the regulation of cell volume³. The enzyme consists of a large catalytic subunit (α), with a relative molecular mass (M_r) of 110,000, and a smaller glycoprotein subunit (β), of unknown function, with an M_r of ~55,000 (ref. 4). The ATP-binding site⁵ and the phosphorylation site⁶ are both located on the cytoplasmic side of the α -subunit and a binding site for cardiac glycosides such as digoxin and ouabain is located on the extracellular side⁷. There is much interest in the location and structure of the ouabain binding site because it is thought to be the pharmacological receptor site for the digitalis class of drugs⁷⁻¹¹.

Considerable effort has been directed at determining the molecular mechanism of active cation transport. The catalytic subunit can exist in at least two conformational states and it is apparently during the transition between these states that Na^+ and K^+ traverse the membrane^{12,13}. This conformational transition is extensive and seems to involve at least 80 amino acids which undergo a transition between α -helix and β -sheet¹⁴ during each cycle of phosphorylation and dephosphorylation. Because the binding of Na^+ and K^+ , cardiac glycosides and ATP all influence the conformational state of the enzyme¹³, there seems to be communication throughout the molecule, involving cytoplasmic, transmembrane and extracellular domains. In addition to the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ there are several other eukaryotic plasma membrane proteins involved in primary active transport. These include the Ca^{2+} -ATPase¹⁵, the $(\text{H}^+ + \text{K}^+)\text{ATPase}$ ¹⁶, and an H^+ -ATPase found in fungi¹⁷. The available data suggest similarities in their structures and enzymatic mechanisms and there is evidence that they have evolved from a common ancestor¹⁸. Structural comparisons of these transport ATPases may help to elucidate their function.

Here we describe the isolation and characterization of a complementary DNA clone containing the entire coding region of the messenger RNA for the catalytic subunit of the sheep kidney $(\text{Na}^+ + \text{K}^+)\text{ATPase}$. The complete amino-acid sequence has been deduced and a comparison with the Ca^{2+} -ATPase sequence reveals extensive regions of homology. This and some other comparisons have allowed us to identify several possible functional domains, including a conserved sequence of ~60 amino acids which is likely to be a major component of the energy transduction system. This sequence spans the membrane and is bounded at one end by the phosphorylation site and at the other by the apparent ouabain binding site.

Isolation of cDNA clones

Sixteen tryptic peptides of the sheep kidney $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ α -subunit have been characterized previously¹⁹ and two of these peptides, T14 and T16, were used to derive oligonucleotide hybridization probes. T14, located at the ATP-binding site, contains the sequence Met-Lys-Gly-Ala-Pro and T16 contains the sequence Val-Phe-Gln-Ala-Asn-Gln-Asp. A 14-base probe, 5' G-G-(A,G,T,C)-G-C-(A,G,T,C)-C-C-(C,T)-T-T-C-A-T 3', which was a mix of 32 oligonucleotides, and a 20-base probe, 5'T-C-(T,C)-T-G(A,G)-T-T(A,G,T,C)-G-C(T,C)-T-G-(A,G)-A-A-(A,G,T,C)-A-C3', which was a mix of 256 oligonucleotides, were derived from T14 and T16, respectively. A library of 50,000 colonies constructed with size-selected cDNA prepared from sheep kidney poly(A)⁺ RNA was screened separately with each probe. Twelve colonies gave signals with both oligonucleotides and plasmid DNA from these clones was isolated and analysed by restriction mapping and blot hybridization analysis.

The size of the cDNA inserts ranged from 2.30 to 3.65 kilobases (kb) (data not shown). Eleven of the twelve clones had a 1.85-kb *Pst*I fragment, which corresponds to the 3' end of the mRNA, and a variable *Pst*I fragment ranging in size from 0.45 to 1.8 kb, which corresponds to the 5' end of the mRNA. In each case a 0.44-kb *Bam*HI restriction fragment gave strong signals with both oligonucleotides when analysed by blot hybridization. To confirm the identity of these clones we first determined the nucleotide sequence of αNKA1 in the region containing homology to the hybridization probes (see Fig. 1a for the restriction map of αNKA1 and the sequencing strategy). The nucleotide sequences encoding T14, T16 and several other known tryptic peptides of the sheep enzyme were identified. All 16 of the known tryptic peptides have been identified in the full sequence described below.

Nucleotide and amino-acid sequences

The complete nucleotide sequence for the largest clone, αNKA1 , and the deduced amino-acid sequence are shown in Fig. 1b. It contains 254 bases of 5'-untranslated sequence, a 3,063-base open reading frame and 340 bases of 3'-untranslated sequence. An additional 10 bases of 5'-untranslated sequence were determined from clone αNKA2 and are included in Fig. 1b. The 3'-untranslated region contains the canonical polyadenylation signal²⁰ 23 bases upstream from the poly(A) tract. The position of the poly(A) tract was confirmed by sequencing the 3' end of αNKA3 , which contains a 14-residue poly(A) tract in this position rather than the three A residues seen in αNKA1 .

A striking feature of the 5'-untranslated region is the G + C-richness (72%) with an abundance of CpG dinucleotides, which are under-represented in the vertebrate genome as a

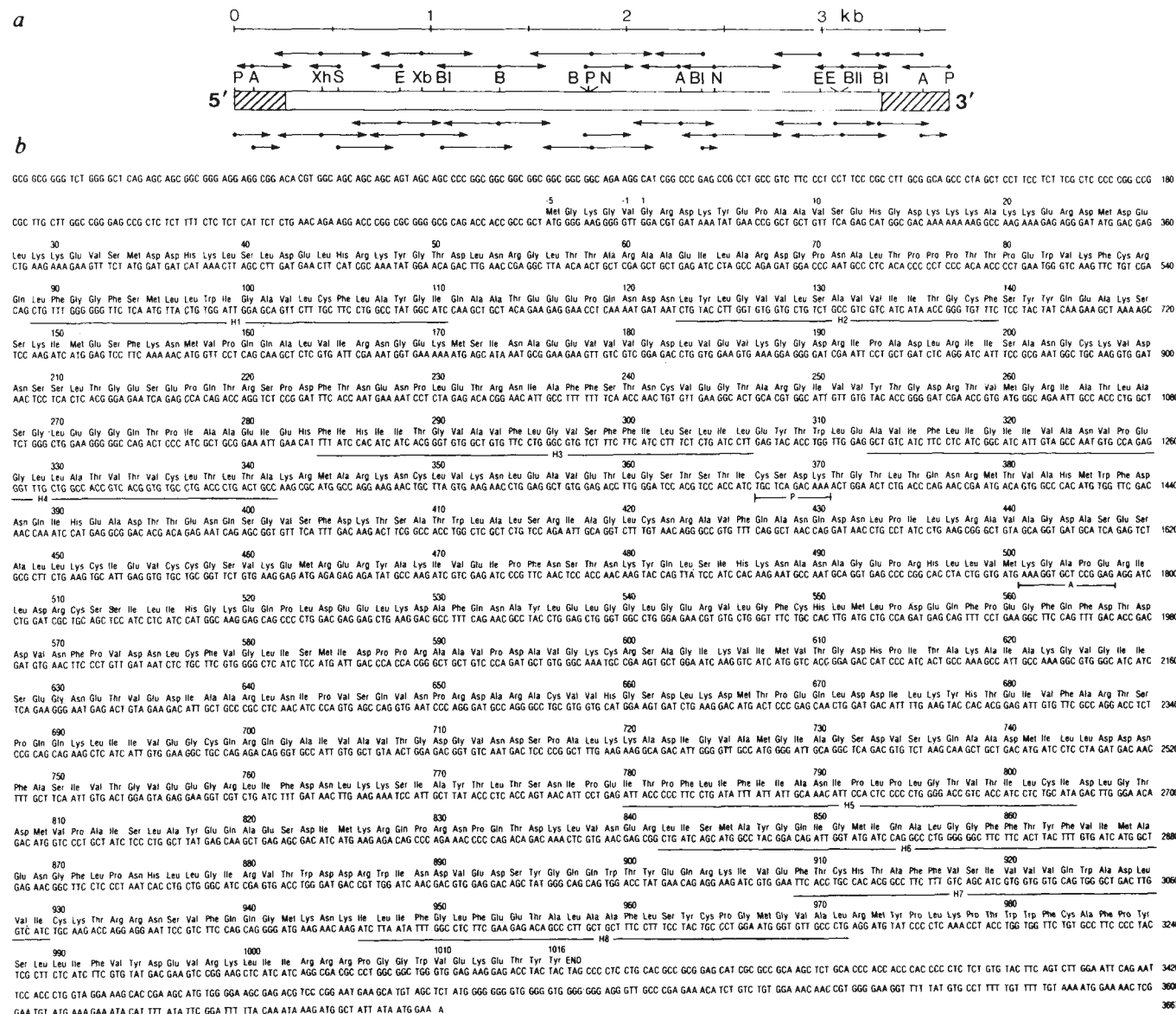


Fig. 1 Sheep kidney ($\text{Na}^+ + \text{K}^+$)ATPase α -subunit cDNA. *a*, Restriction map of clone pNKA1 and sequencing strategy. Only the restriction sites (P, *Pst*I; A, *Aat*I; Xh, *Xho*I; S, *Sal*I; E, *Eco*RI; Xb, *Xba*I; B1, *Bgl*I; B, *Bam*HI; N, *Nco*I; B11, *Bgl*II) relevant to the sequencing strategy are shown. The extent and direction of sequencing are indicated by arrows (lower arrows, coding strand; upper arrows, noncoding strand). Hatched areas represent the 5' and 3' untranslated regions. *b*, Nucleotide sequence of clone pNKA1 and deduced amino-acid sequence. The first 10 nucleotides at the 5' end were determined from clone pNKA2 which extended slightly beyond the 5' end of pNKA1. Amino acids are numbered starting at the N-terminal Gly of the mature protein and are preceded by five amino acids occurring in the primary translation product. The eight major hydrophobic regions (H1-H8) are underlined. Brackets indicate the putative phosphorylation site (P), centred around Asp 369, and a portion of the ATP-binding site region (A), which includes Lys 501.

Methods. Poly(A)⁺RNA was isolated^{46,47} from sheep kidney. cDNA was synthesized by the procedure of Gubler and Hoffman⁴⁸, size-selected by electrophoresis in agarose and capture of the 2,100–4,500-base-pair fraction on NA45 paper (Schleicher & Schuell), tailed with dCTP and annealed⁴⁹ with G-tailed *Pst*I-cut pBR322. *E. coli* RRI cells were transformed with 60 ng of cDNA and grown on agar plates containing tetracycline. Colonies were transferred to nitrocellulose master filters which were used to prepare two replicas⁵⁰ on Zeta Bind membranes (AMF Cuno). Following additional growth on tetracycline and amplification on chloramphenicol, the filters were processed by the procedure of Geyer and Johnson⁵¹. Filters were hybridized successively with two oligonucleotide probes (see text for composition of probes) which were 5' end labelled using *T*₄ polynucleotide kinase and [γ -³²P]ATP. Conditions for hybridization, washing and autoradiography were basically as described by Boyd *et al.*⁵². DNA sequencing was performed by the chemical cleavage method of Maxam and Gilbert⁵³. Restriction fragments were labelled either at the 5' end using [γ -³²P]ATP and *T*₄ polynucleotide kinase, or at the 3' end using the Klenow fragment of DNA polymerase I and [α -³²P]dNTPs.

whole²¹. The phenomenon of CpG suppression is attributed to the effects of methylation and subsequent mutation that causes a decrease in CpG content and a corresponding increase in the number of TpG and CpA dinucleotides²¹. In the case of the ($\text{Na}^+ + \text{K}^+$)ATPase mRNA, there is no apparent CpG suppression in the 5'-untranslated region; the CpG content is 90% of the value expected based on nucleotide composition, whereas the TpG + CpA content is 87% of the expected value. However,

CpG suppression is apparent in the coding region, where the CpG content was 46% of the expected value against 135% for TpG + CpA. Recent evidence suggests that G + C-richness may be a common feature of the 5' regions of housekeeping genes^{22,23} and the present results support that view. As suggested by Bird²⁴, it may be useful to consider an evolutionary perspective when assessing the significance of these G + C-rich tracts. As these regions have escaped CpG suppression, their functional role

a

NKA 173 INAEVVVGDVLEVKGGDRIPADLRISANGC·KVDNSSLTGES 215 233 RNIAFFSTNCVEGTARGIVVYTGORTVMGRITATSLGLEGGQTP 276
 CA 140 IKAKDIYPGDIVEIAVGDKVPADIRLTSIKSTTLRVDSILTGES 184 205 KNMLFSGTNIAAGKAMGVVATGVNTEIGKIRDEMVAETEERTP 248

NKA 322 VANVPEGLLATVTVCLTLTAKRMARKNCLVKNLEAVETLGSTSTICSOKTGTLTQNRMTVAHM 384 499 VMKGAPERILDRCSILINGKEQPLDEELK 528
 CA 304 VAAIPEGLPAVITTCALGTRRMAKKNNAIVRSLPSVETLGCTSVICSOKTGTLTQNMVSCRM 366 512 FVKGAPGVDRCTHIRVGSTKVPMTAGVK 541

NKA 575 NLCFVGLISMIDPPRAAVPOAVGKCRSAGIKVIMVTGDNHPITAKATAKGVGI 626 683 FARTSPQQLIIVEGCQROGAIVAVTGDDVNDSPALKKADI 723
 CA 589 NLTFVGCVMLOPPRIEVAASSVKLCROAGIRVIMITGDNKGTAVAICRRIGI 640 675 FARVEPSHKSKIIVEFLQSFDEITAMTGDGVNDAPALKKAEI 715

NKA 724 GVAMGJAGSDVSKOAAOMILLDDNFASIVTGVVEEGLIFDNKKSIAYTLTNSNIP 779 807 TDMVPAISLAYEQAESDIMKROPNNPDTDKLVNERLIS 844
 CA 716 GIAMG-SGTAVAKTASEMVLADDNFSTIVAEEGRAIYNNMKQFIYRLISSNVGE 770 798 TDGLPATALGFNPPDLIMNKPNNP---K---EPLIS 829

NKA 975 PLKPTIWWFCAPFYSLLIFVYDEVKLIIR 1003
 CA 960 PLNVTWLMLVKISLPVILMDETLKFFAR 988

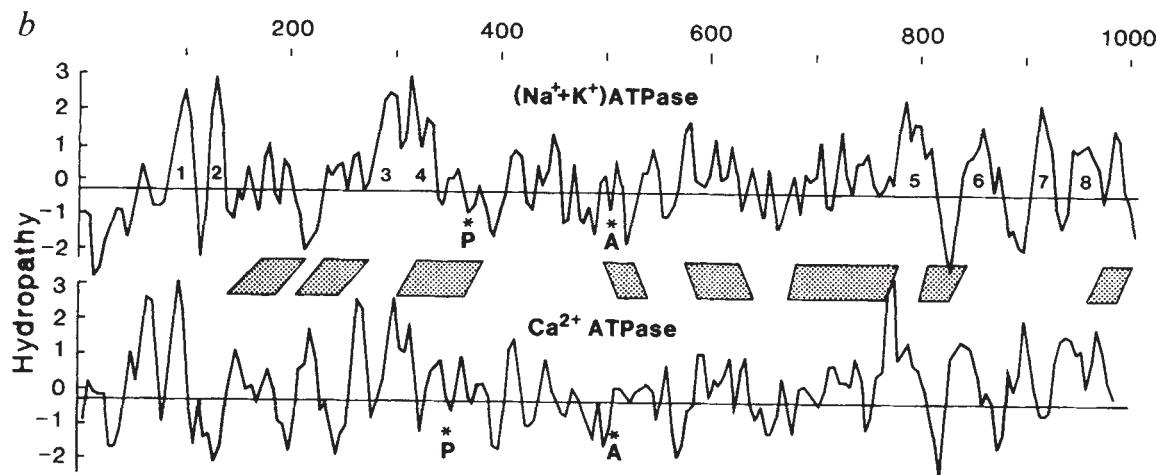


Fig. 2 Comparison of the amino-acid homology and hydropathy plots of the sheep kidney $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ α -subunit and the rabbit cardiac $\text{Ca}^{2+}\text{-ATPase}$. **a**, Amino-acid homology between the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ (NKA) and the $\text{Ca}^{2+}\text{-ATPase}$ (CA). The sequences compared are those with $>30\%$ homology. Homologous residues are indicated by an asterisk; gaps have been included to maximize homology. **b**, Hydropathy plots of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ (top panel) and $\text{Ca}^{2+}\text{-ATPase}$ (bottom panel). Hydrophobic regions are above the x axis and hydrophilic regions below it. The parallellograms indicate the regions of homology shown in **a** and their relative positions along the peptide chains. The phosphorylation site is indicated by P and the ATP binding site by A. The numbers indicate the eight major hydrophobic regions of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$. Homology comparisons and hydropathy analyses were performed using the International Biotechnologies sequence analysis programs. The hydropathy program is based on the procedure of Kyte and Doolittle³¹.

may have little to do with methylation (which may be of recent evolutionary origin and confined to vertebrates²⁵) but instead could be part of an evolutionarily older regulatory process which operates in both vertebrates and invertebrates.

The identification of the translation initiation site is unambiguous as it is the first AUG codon; it is preceded by 264 bases of G+C-rich sequence and lies within the consensus sequence for initiation described by Kozak²⁶. The initiation methionine is located five amino acids upstream from the known N-terminal peptide of the protein¹⁹. Thus, the primary translation product does not contain a hydrophobic N-terminal signal peptide. Only a five-amino-acid sequence, Met-Gly-Lys-Gly-Val, is removed to yield the N-terminus of the mature protein, which has 1,016 amino acids and an M_r of 112,177. The C-terminal amino acids, Thr-Tyr-Tyr-COOH, are identical to those of the dog kidney enzyme²⁷.

Several considerations lead us to conclude that the Asp that is phosphorylated during the catalytic cycle²⁸ is at position 369. The most compelling evidence is its location within an extended region of homology with the phosphorylation site region of the $\text{Ca}^{2+}\text{-ATPase}$ ²⁹ (see below). Furthermore, the surrounding sequence, Cys-Ser-Asp-Lys (Fig. 1b), is consistent with that determined by Bastide *et al.*⁶ and lies within the region predicted on the basis of experiments using proteolytic digestion (reviewed in ref. 13). Several laboratories have identified a peptide containing a Lys residue which reacts with fluorescein isothiocyanate (FITC) and which forms a part of the ATP-binding site^{5,30}. This FITC peptide corresponds to residues 496–506 (Fig. 1b). The

reactive residue, Lys 501, is located 132 amino acids away from the phosphorylation site.

There are eight major hydrophobic sequences (H1–H8), ranging in size from 17 to 29 amino acids (see Figs 1b, 2b). Based on the hydropathy values of membrane-spanning segments analysed by Kyte and Doolittle³¹, it is likely that each traverses the membrane.

Homology with $\text{Ca}^{2+}\text{-ATPase}$

A comparison of the amino-acid sequence and hydropathy plot of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ α -subunit with those of the $\text{Ca}^{2+}\text{-ATPase}$ ³² is shown in Fig. 2. The overall structural organization of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ is strikingly similar to that of the $\text{Ca}^{2+}\text{-ATPase}$. The two proteins are virtually identical in size and in the location of their phosphorylation and ATP-binding sites and each of the eight large hydrophobic domains of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ has its counterpart in the $\text{Ca}^{2+}\text{-ATPase}$ (Fig. 2b). Except for three regions where ≥ 20 amino acids seem to have been inserted into or deleted from one or the other protein, matching structural features occur throughout the peptide chains. These proteins have clearly evolved from a common ancestor.

Several major regions of amino-acid homology (Fig. 2a) are located between H2 and H3 and between H4 and H5. Experimental evidence, both for the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ ³³ and for the $\text{Ca}^{2+}\text{-ATPase}$ ¹⁵, suggests that these areas are on the cytoplasmic side of the membrane. The greatest homology is near the phosphorylation site. A 10-amino-acid sequence, which includes Asp

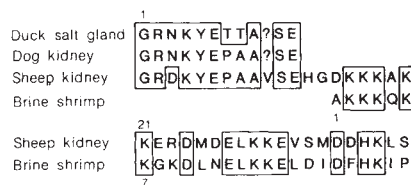


Fig. 3 Comparison of the N-terminal amino-acid sequences of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ α -subunit of several species. Identical residues are boxed. Duck salt gland data from ref. 35; dog kidney data from ref. 34; brine shrimp data from ref. 36.

369, is identical in the two proteins. The homology ends abruptly at Met 384, where the hydrophathy values become hydrophilic for the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ but are somewhat hydrophobic for the $\text{Ca}^{2+}\text{-ATPase}$. An insertion or deletion may have occurred here because the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ has 32 fewer amino acids between the phosphorylation site and the ATP-binding site. There is only a small region of homology at the ATP-binding site but several longer regions of homology occur nearer the C-terminus, including an interesting hydrophilic domain at residues 807–844. If H5 and H6 span the membrane, this sequence of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ would be on the exterior of the cell, whereas for the $\text{Ca}^{2+}\text{-ATPase}$ it would be inside the sarcoplasmic reticulum.

There is no clear evidence regarding the location of the C-terminus for either the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ or the $\text{Ca}^{2+}\text{-ATPase}$. However, as the last 20 amino acids of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ are fairly hydrophilic it is more likely to be located on the cytoplasmic side of the membrane. Also, a cytoplasmic location would be necessary if all four hydrophobic domains in the C-terminal half of the protein span the membrane. The hydrophathy values³¹ of these segments and the corresponding segments of the $\text{Ca}^{2+}\text{-ATPase}$ suggest that this is the case.

N-terminal domains

The N-terminal region of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ is strongly hydrophilic and does not have a homologous counterpart in the $\text{Ca}^{2+}\text{-ATPase}$. If the first major hydrophobic domains of the two proteins are aligned it seems that the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ has ~35 additional amino acids at its N-terminus (see Fig. 2b). Figure 3 shows a comparison of the N-terminal sequences of mammalian, avian and crustacean $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ α -subunits. The similarity between the sheep and dog kidney

enzymes³⁴ and that of the duck nasal salt gland³⁵ was noted previously but the crustacean (brine shrimp) sequences were thought to be very different³⁶. However, we find that this is not the case. Although the first 14 amino acids in the sheep enzyme are absent from the brine shrimp enzyme, there is considerable homology beyond that point, particularly the conservation of several lysine residues. The brine shrimp isoform that most closely resembles the sheep enzyme has 9 lysines among the first 26 amino acids³⁶. Eight of these residues are present in the sheep enzyme; an Arg is present at the remaining position. This is quite striking because the evolutionary divergence of these two species occurred over 400 Myr ago.

Several lines of evidence suggest that this region influences the cation-dependent equilibrium between the E1 and E2 conformational states³³. A conformation-dependent trypsin site ~20 amino acids from the N-terminus is cleaved rapidly in 10 mM NaCl, yielding an N-terminal Ala, but is inaccessible in KCl^{27,33}. The small fragment removed contains a histidine which may be involved in the cation effect on the E1–E2 equilibrium^{33,37}. This trypsin site could be at Lys 18, as it is followed by an Ala and preceded by a His at position 13. The brine shrimp enzyme, which is exposed to a very different salt environment from that of mammals and birds, lacks the short sequence that contains His 13. An interesting possibility is that the N-terminal hydrophilic domain, which contains many charged residues including the highly conserved Lys residues (Fig. 3), controls the passage of Na^+ and K^+ ions to and from cation-binding sites on the cytoplasmic side of the protein. This possibility is consistent with the lack of homology in this region between the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ and the $\text{Ca}^{2+}\text{-ATPase}$.

The N-terminus of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ ³⁸, like the $\text{Ca}^{2+}\text{-ATPase}$ ³⁹, is located on the cytoplasmic side of the membrane. As the region between H2 and H3 is also cytoplasmic, H1 and H2 probably span the membrane and the hydrophilic junction between them should be located on the extracellular side of the membrane. It has been suggested that transmembrane sequences in this region form part of an ion channel³³. The presence of several acidic residues between H1 and H2, which could serve as cation-binding sites on the extracellular surface, is consistent with this possibility. Also, Ca^{2+} -ionophore activity has been demonstrated for the corresponding region of the $\text{Ca}^{2+}\text{-ATPase}$ ⁴⁰.

Energy transduction region

One of the most interesting sections of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ is the region which contains the phosphorylation site and hydrophobic domains H3 and H4 (Fig. 2b). A proteolytic fragment

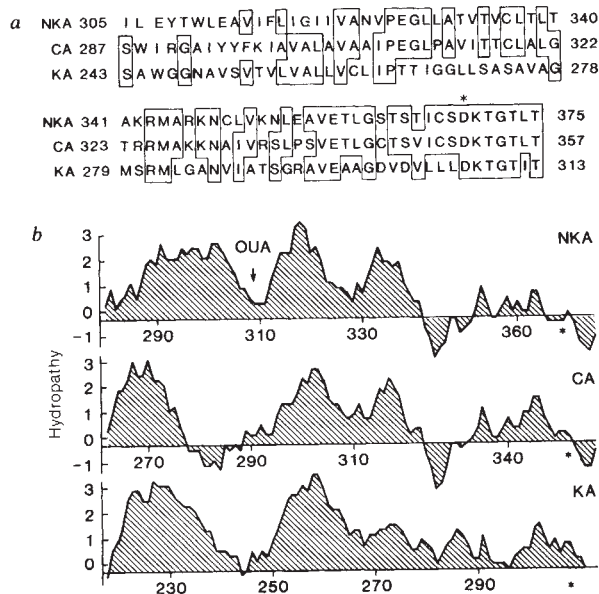


Fig. 4 Energy transduction region and ouabain binding site. **a**, Comparison of the amino-acid sequence of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ (NKA) with the corresponding sequences of the $\text{Ca}^{2+}\text{-ATPase}$ (CA) and the $\text{K}^+\text{-ATPase}$ B protein (KA) of *E. coli*¹⁸. The sequences compared extend from Ile 305 of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$, which is on the N-terminal side of Asp 369, the phosphorylation site (indicated by the asterisk). Identical residues are boxed. **b**, Comparison of the hydropathy plots for the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ (top panel) extending from Glu 280 to Thr 375, which includes H3 and H4 and the phosphorylation site, indicated by the asterisk, with those of the corresponding region for the $\text{Ca}^{2+}\text{-ATPase}$ (middle panel) and the $\text{K}^+\text{-ATPase}$ B protein of *E. coli* (bottom panel). The probable location of the ouabain binding site is indicated. The hydropathy plots are not condensed as in Fig. 2b; nine residue averages for each amino acid have been plotted³¹.

that maps to this region contains membrane-embedded sequences and includes both the phosphorylation site and a site which reacts with a ouabain photoaffinity derivative³⁸. The accessibility of trypsin and chymotrypsin sites at the N-terminal boundary of this peptide, and a trypsin site at the C-terminal boundary, are dependent on the conformation of the enzyme (reviewed in ref. 33). Possible locations of these proteolytic sites are, respectively: Arg 262, which would yield the expected N-terminal isoleucine²⁷; Phe 284, the first residue of H3; and Arg 423, which would yield the expected N-terminal Ala²⁷.

A region located between the trypsin sites was compared with similar sequences of the Ca²⁺-ATPase and the K⁺-ATPase B protein of *Escherichia coli*¹⁸. When the full hydroplot for the K⁺-ATPase B protein was compared with that of the (Na⁺ + K⁺)ATPase (data not shown), the only major hydrophobic domains which seemed to be analogous were those next to the phosphorylation site. Furthermore, there was a region of amino-acid homology around the phosphorylation site that extended into H4. This is of interest because H4 is the only major hydrophobic domain that exhibits strong amino-acid homology between the (Na⁺ + K⁺)ATPase and the Ca²⁺-ATPase, and because of its proximity to the phosphorylation site it would be a likely pathway for transmitting conformational changes into the intramembrane regions of the protein. The amino-acid homology for this section of all three enzymes is shown in Fig. 4a and the hydropathy plots for sequences including H3 and extending through the phosphorylation site are shown in Fig. 4b. The similarity in their hydropathy plots and the amino-acid homology between these eukaryotic and prokaryotic ATPases suggests that the sequence extending from the phosphorylation site into H4 may be an energy transduction pathway⁴¹ into the transmembrane domains involved in cation transport.

Cardiac glycoside binding site

An attractive possibility for the location of the ouabain binding site is the junction between H3 and H4. Studies with anthrolyl-ouabain indicate that the binding site is relatively hydrophobic, shielded from water and is close to a Trp residue⁴². This observation prompted the development of a ouabain photoaffinity derivative which is activated by energy transfer from an excited Trp residue; this derivative was incorporated exclusively into the N-terminal half of the protein⁴³. Similar results were obtained with an alkylating ouabain derivative⁴⁴. The ouabain binding site in this portion of the molecule seems to be localized to the H3-H4 region, but binding has also been demonstrated in the C-terminal region³⁸. It has been suggested that this additional site, which could be at the H5-H6 junction or the H7-H8 junction, is not specific for the steroid-like portion

of the molecule⁴³.

If the path of the polypeptide chain through the membrane is as suggested here, then there are just four short extracellular sequences. Only the H3-H4 junction, which includes residues 307-312 and probably some adjacent residues, seems to have all the properties predicted for the ouabain binding site. First, it is relatively hydrophobic yet should not be embedded in the membrane as it contains two glutamate residues, a Trp and a Tyr. Trp and Tyr, although hydrophobic in some respects, are infrequently buried⁴⁵. Second, the presence of a Trp is expected based on the studies described above and Trp 310 is the only such residue found in the probable extracellular regions of the protein. Third, this site is not homologous with the corresponding region of the Ca²⁺-ATPase, which does not interact with cardiac glycosides. Fourth, given the inhibitory effects of cardiac glycosides on enzyme activity⁷, this site is ideally situated with respect to the proposed energy transduction region and phosphorylation site.

Conclusions

The probable structure of the catalytic subunit of the (Na⁺ + K⁺)ATPase, deduced from the present work and the studies discussed above, is as follows. The protein consists of 1,016 amino acids and has eight transmembrane domains. Much of the protein, including the hydrophilic N-terminal domain, the sequences between H2 and H3 and those between H4 and H5, is located on the cytoplasmic side of the membrane. The C-terminus and the region between H6 and H7 are also likely to be cytoplasmic. Very little of the protein is extracellular.

As well as the phosphorylation site and the ATP-binding site, several possible functional domains have been identified. The hydrophilic N-terminal domain might function on the cytoplasmic side as an ion-selective barrier which controls access to cation-binding sites. Hydrophobic domains H1 and H2 may form part of a cation channel and the hydrophilic junction between them is a likely candidate for extracellular cation-binding sites. A relatively hydrophobic extracellular cleft between H3 and H4 has the characteristics expected for the digitalis binding site and is connected to the phosphorylation site by a sequence which may be an important component of the energy transduction system.

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Amino-acid sequence of a $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence

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We have cloned and sequenced complementary DNA encoding a Ca^{2+} -ATPase of rabbit muscle sarcoplasmic reticulum. We propose a model of the protein which has 3 cytoplasmic domains joined to a set of 10 transmembrane helices by a narrow, penta-helical stalk. In this model, ATP bound to one cytoplasmic domain would phosphorylate an aspartate in an adjoining cytoplasmic domain, inducing translocation of Ca^{2+} from binding sites on the stalk.

THE $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase (Ca^{2+} -ATPase) of rabbit skeletal muscle sarcoplasmic reticulum, first isolated in 1970 (ref. 1), is a mammalian ion-transport enzyme which has been extensively investigated at the molecular level². The membrane-bound enzyme is cleaved instantly by trypsin (at site T_1) into two fragments of equal length, A and B; fragment A is cleaved more slowly (at site T_2) into unequal subfragments A_2 and A_1 (ref. 3). Functional sites have been associated with these fragments, each of which is independently anchored in the membrane². More detailed information on the topography of the enzyme and on the localization of active sites has been obtained from the amino-acid sequence of five cytoplasmic segments of the enzyme which include the amino-terminus (sequence 1), the

carboxy-terminus (sequence 5) and three long, internal segments (sequences 2–4)⁴. Hydrophobic transmembrane sequences have not been obtained, however, leaving a gap in our understanding of the structures through which Ca^{2+} ions are bound and transported across the membrane.

The need to obtain the full sequence of the Ca^{2+} transport enzyme, together with the desirability of obtaining new information about genes for homologous proteins and the expression of these genes during development, has led us to clone complementary DNA encoding two forms of the Ca^{2+} -ATPase. The characterization of one of these cDNA clones has permitted us to determine its complete primary structure and to predict its secondary and tertiary structure.

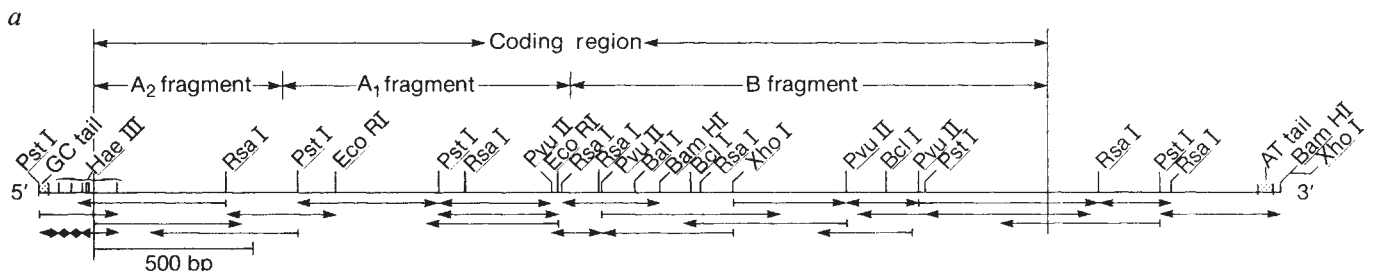


Fig. 1a. Sequencing strategy for cloned cDNA encoding a Ca^{2+} -ATPase protein of rabbit muscle sarcoplasmic reticulum. The restriction map displays six-base restriction endonuclease sites and those four-base restriction sites that were used in sequencing. The coding region is divided into three segments, A_2 , A_1 and B, which correspond to the A and B fragments generated by brief tryptic digestion of the fast twitch skeletal ATPase protein in intact sarcoplasmic reticulum³. **b.** Nucleotide and deduced amino-acid sequence of the Ca^{2+} -ATPase. The amino-acid sequence of the clone bears 84% identity with the fast skeletal ATPase⁴ and identity with a sequence of 17 amino-acid residues ((R)TSMKMFVKGAPEGVIDR beginning at residue 505) found in the Ca^{2+} -ATPase from canine cardiac sarcoplasmic reticulum (Briggs, F. N. *et al.*, in preparation). Nucleotide residues are numbered positively in the 5' to 3' orientation beginning with residue 1 of the ATG codon for the initiator methionine and ending with the last residue before the poly(dA) tail. They are numbered negatively beginning from the 5' side of residue 1 to the beginning of the poly(dG) tail (added to the 5' end of the cDNA before its incorporation into the vector pCD-X³⁶). The deduced amino-acid sequence is shown below the nucleotide sequence. Amino-acid residues are numbered beginning with the initiator methionine below the residues on the left-hand of each row. Polar segments, of sufficient length to be transmembrane, are underlined. Extensions of transmembrane segments and potential transmembrane segments (see Fig. 2) are underlined with a broken line. The major tryptic sites (T_2 and T_1), the sites of phosphorylation (P) and the site of FITC binding (F) are indicated beneath the appropriate amino acids.

Methods. Total RNA was extracted from skeletal muscle of 1–3-day-old rabbits as described previously³⁷, with the following modifications: SDS and diethyl pyrocarbonate were absent from all solutions and RNA was extracted with CHCl_3 /isoamyl alcohol (4:1) before a single step of chromatography on an oligo(dT)-Sepharose column. A series of cDNA libraries was constructed in the vector pBR322 by standard procedures involving first-strand synthesis by reverse transcription of oligo(dT)-primed mRNA, second-strand synthesis with the Klenow fragment of DNA polymerase, removal of the hairpin loop with S_1 nuclease, tailing with deoxycytidine and ligation of the double-stranded cDNA with Pst I-cleaved, oligo(dG)-tailed pBR322. The first library of 3,500 clones was probed with a group of four synthetic oligomeric deoxynucleotides of composition 5'-GC⁺TACAT⁺GAACCA-3 based on the amino-acid sequence (W)FMYA recently determined for a hydrophobic peptide in the B fragment of the Ca^{2+} -ATPase³⁸. This screening yielded the first fast skeletal muscle ATPase clone, pFA1, containing 75 bp of coding sequence lying within a 234-bp Pvu II/ Pvu II fragment near the 3' end of the fast skeletal muscle ATPase clone (the homologous Pvu II/ Pvu II fragment is present in the clone described in Fig. 1). A fragment of 71 bp was cut from pFA1 with Sau 3a and Hae III and used to probe a second library of 50,000 clones yielding six clones of fast skeletal ATPase and two cross-hybridizing clones, pCA1 (1,900 bp) and pCA2, which contained all of the residues in pCA1 but terminated earlier in the 3'-untranslated region. These clones were sequenced and the 234-bp Pvu II/ Pvu II fragments from both groups of clones were used to probe a library of 1.6×10^6 clones in the vector pCD-X using the method of Okayama and Berg³⁶. This library yielded 3 fast skeletal muscle clones and 16 cross-hybridizing clones. The longest cross-hybridizing clone, pCA3 (3,722 bp), was restriction-mapped as illustrated above and clones pCA3 and pCA4 (3,602 bp) were used for sequence analysis. Restriction endonuclease fragments were subcloned into the vectors pEMBL8 and pEMBL9 to obtain sequences in both orientations³⁹. Sequences initiated at sites other than restriction sites were obtained from the shorter clones that terminated in regions inaccessible by suitable restriction endonuclease fragmentation. Production of single-stranded DNA suitable for DNA sequencing was induced by superinfection with the phage IR1 (ref. 39). Single-stranded DNA was sequenced by the dideoxy method of Sanger *et al.*⁴⁰.

initiator Met codons in the first 128 nucleotides upstream of the ATG in our ATPase transcript suggests that this region is untranslated, consistent with our earlier observations¹¹ that the fast skeletal muscle Ca^{2+} -ATPase is synthesized without an amino-terminal signal sequence.

We do not yet know whether the 128 nucleotides upstream of the first Met codon represent all, or only part, of the 5'-untranslated region of the ATPase transcript. Northern blot analysis of neonatal rabbit muscle messenger RNAs with our cDNA probes showed a hybridizable transcript of ~3.7 kilobases, suggesting that the cDNA clones are very close to full length. The 5'-untranslated sequence contained 65 G, 44 C, 16 A and 3 T residues. Thus, the nucleotide content of this sequence resembled the 5'-untranslated region of the $(\text{Na}^+ + \text{K}^+)$ ATPase gene transcript published elsewhere in this issue¹², but was even more G+C-rich (85%).

The 3'-untranslated region terminated in a poly(dA) tail. The sequence upstream of the tail did not contain a perfect AATAAA polyadenylation signal¹³, but the sequence AATACAA terminated 21 bases before the poly(dA) tail and was followed closely by the sequence TGTGATGC, which is very similar to the consensus sequence YGTGTTY found between many polyadenylation signals and their poly(A) tails¹⁴.

A sequence beginning 51 bases downstream of the stop codon (nucleotides 3,045–3,074) contained an interesting homology with the core sequence of the murine IgG enhancer sequence, although loop-outs would be necessary in order to achieve maximal homology. Our sequence GTGCGTTTTAGCAACACACCTGCCAGCCC (nucleotides 3,045–3,074) resembled the sequence GTGGTTTGAACACTCTGTCAGCCC reported by Hayday *et al.*¹⁵ as comprising part of an enhancer sequence.

Homology between ATPases

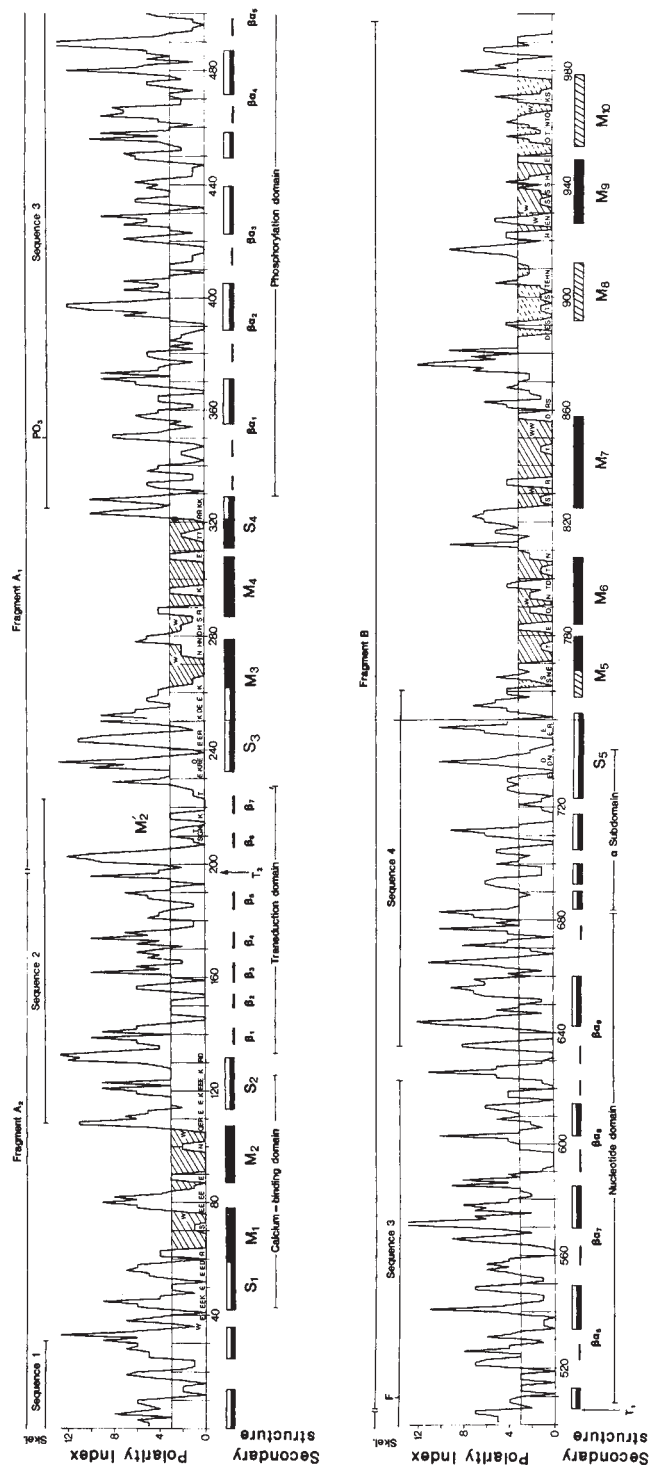
Homology between the amino-acid sequences of the extramembranous segments of the Ca^{2+} -ATPase and the K^+ -ATPase of *Escherichia coli* has already been pointed out¹⁶. There was ~50% homology in some limited regions covering a total of ~180 residues. Note that there is little or no homology in the

transmembrane regions beyond a general hydrophobicity, but that the location of these hydrophobic sequences in the overall sequence is very similar in the two ATPases. The regions of homology with the $(\text{Na}^+ + \text{K}^+)$ ATPase¹² were more extensive and included the transmembrane segments M_4 , M_6 and M_7 (see Fig. 2). The first transmembrane segment, M_1 , is interesting in that both Ca^{2+} and $(\text{Na}^+ + \text{K}^+)$ ATPase sequences showed separate homologies with the M_1 section of the K^+ -ATPase while showing little resemblance to each other.

Secondary and tertiary structure

In the absence of a database of membrane proteins of known structure, empirical prediction of their secondary structure is unreliable¹⁷ as strongly hydrophobic segments are almost invariably predicted as β -strands. Therefore, we have identified the

Fig. 2 Polarity profile and secondary structure analysis of the Ca^{2+} -ATPase aligned with the tryptic fragments and the previously determined major sequences of the fast skeletal muscle ATPase. The top line ('fragment') represents tryptic fragments (A_2 , A_1 and B) of the fast skeletal muscle ATPase³. The second line ('sequence') indicates previously determined major sequences of the fast skeletal muscle ATPase⁴, showing phosphorylation and fluorescein-reactive sites. The polarity index of amino-acid triplets is shown below these two lines (only polar residues score: S, T = 1; N, Q, H = 2; D, E, K = 3; R = 4). The plot is designed to emphasize polar interruptions of potential transmembrane helices. In a small helical bundle any polar interruption of >2 residues is likely to force at least one polar residue out into the hydrophobic lipid phase. For this reason, penalty points are added to the triplet score equal to the lowest score of the two outer residues. Any run of 21 residues rising nowhere above polarity 3 is a putative transmembrane sequence. Individual polar residues within putative transmembrane stretches are shown, facilitating evaluation of their influence. Other individually noted residues are charged residues in the α -helices that link the globular domains to the membrane, and tryptophans (changes in tryptophan fluorescence have been used to monitor conformational changes in the reaction cycle). We note that 11 out of 13 residues are located in transmembrane segments). Secondary structure is identified by the template fitting procedure of Taylor and Thornton¹⁹ and applied to long extramembranous segments. ■, Amphipathic helix; —, β -strand; ▬, hydrophobic transmembrane helix. The nomenclature for structural domains is shown at the bottom of the figure. The transmembrane domain consists of hydrophobic helices M_1 – M_{10} ; the stalk of amphipathic connecting helices S_1 – S_5 ; the transduction domain of residues 133–238 (antiparallel β -sheet); the phosphorylation domain of residues 330–505 (parallel β -sheet); the nucleotide-binding domain of residues 506–680 (parallel β -sheet); and the α -helical sub-domain of residues 681–740.



include the amino-terminus as a small additional domain. Because the connecting segments will form the narrow neck of the ATPase molecule, which can be seen in electron micrographs of negatively stained vesicles³⁰, we refer to them as stalk segments (S_1 – S_5). All but S_4 are strongly predicted to be helices of length ~ 3 nm. When the residues are written on an α -helical net (Fig. 4) the helices are clearly amphipathic, having a high density of charged residues on one face. The highly conserved segment S_4 , which connects M_4 to the phosphorylation site, is different in having a hydrophobic amino-terminus which gives priority to β -strand over α -helix. Nevertheless, a helix is likely to be favoured because of close association of the section with the other four helices of the stalk. Helices S_1 , S_2 and S_3 are notable for their 16 aligned Glu residues (Fig. 3) which must contribute to the Ca^{2+} -binding sites. With one aspartate, they are the only negatively charged groups adjoining the membrane in the A_2 fragment. There is no sequence there or elsewhere in the molecule which resembles the DXDXDX loop characteristic of the EF hand of Ca^{2+} regulatory proteins³¹. Five or six of these Glu residues will be in the lipid polar head group region on the cytoplasmic surface of the membrane, and four of these will be in the bend regions between the S and M helices (Fig. 3). On the luminal side of the M_1 – M_2 loop there is another cluster of four Glu residues (EEGEE), which is a strong candidate for the low-affinity sites from which Ca^{2+} is released into the lumen. We suggest that the low-affinity Ca^{2+} -binding sites on the luminal face are constituted from a completely different set of residues than the high-affinity sites.

Topography of ATPase domains

An outline of the structure of the Ca^{2+} -ATPase can be deduced from electron micrographs showing a globule attached to the membrane by a narrow stalk³⁰. The transmembrane helices are observed on freeze fracture as intramembranous particles³². In our planar diagram (Fig. 4) we suggest that the three extramembranous globular domains form a headpiece which lies on top of a stalk comprised of five helices which are, in turn, adjoined to five transmembrane helices. These transmembrane helices together with five additional helices at the carboxy-terminal end of the molecule, form the intramembranous basepiece.

Our knowledge of domain function, derived from extensive kinetic and labelling studies³³, suggests that all three extramembranous domains are highly interactive. The nucleotide domain is relatively independent of the rest of the molecule in that it can be blocked by FITC without preventing either phosphorylation from inorganic phosphate or Ca^{2+} transport energized by acetyl phosphate³⁴. Nevertheless, it is probably folded beside the other two globular domains, as it has to transfer the γ -phosphate of ATP to the phosphorylation site on the phosphorylation domain. There is also some interaction of this domain

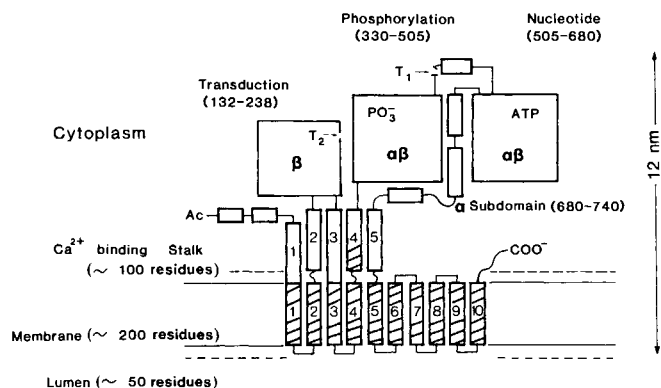


Fig. 4 Assembly of ATPase domains. The predicted arrays of helices and the three major domains are laid out in a planar diagram. In the ATPase molecule the helices would form tight clusters. The ATP-binding domain would be folded beside the other two major domains, permitting interaction among all three and accounting for the approximately triangular molecular profile derived from electron micrographs of ordered arrays by optical diffraction⁴¹ and for the molecular shape deduced from hydrodynamic measurements³⁵ and electron microscopy³⁰.

with the Ca^{2+} -binding site as, for example, bound ATP accelerates the binding of Ca^{2+} , as well as influencing other stages of the reaction cycle³³. The planar diagram, while speculative, represents an advanced model for the secondary and tertiary structure of the Ca^{2+} -ATPase on which refinements and prediction of mechanism may eventually be based.

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Human transforming growth factor- β complementary DNA sequence and expression in normal and transformed cells

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The partial amino-acid sequence of purified human transforming growth factor- β (TGF- β) was used to identify a series of cDNA clones encoding the protein. The cDNA sequence indicates that the 112-amino acid monomeric form of the natural TGF- β homodimer is derived proteolytically from a much longer precursor polypeptide which may be secreted. TGF- β messenger RNA is synthesized in various normal and transformed cells.

TWO different peptides which can induce the reversible phenotypic transformation of mammalian cells in culture are termed transforming growth factor (TGF)¹ α and β . TGF- α is synthesized by various transformed cell lines^{2,3} and competes with epidermal growth factor (EGF) for binding to the same cell-surface receptor². A 50-amino-acid-long TGF- α species has been purified and shown to share sequence homology with EGF⁴. This TGF- α is initially synthesized as part of a 160-amino-acid-long precursor molecule which undergoes NH₂- and C-terminal proteolytic processing to yield the mature peptide^{5,6}.

TGF- β has been isolated from tumour and normal cells and tissues⁷, including kidney⁸, placenta⁹ and blood platelets^{10,11}. The relatively high levels of TGF- β present in platelets, which also contain platelet-derived growth factor (PDGF), have led to the suggestion that TGF- β plays a role in wound healing¹². It is a homodimer of relative molecular mass (M_r) ~ 25,000 (ref. 11) which recognizes a cell surface receptor distinct from the EGF receptor^{13,14}. Treatment of NRK fibroblasts with TGF- β does, however, result in an increase in the number of membrane receptors for EGF¹⁵, which may explain the ability of TGF- β to greatly potentiate transforming activity of EGF and TGF- α on these cells⁷. Moreover, TGF- β alone can induce AKR-2B fibroblasts to form colonies in soft agar¹⁶. In addition to stimulating cell proliferation, TGF- β has recently been demonstrated to inhibit the anchorage-dependent growth of a variety of human cancer cell lines¹⁷. TGF- β may be identical or very similar to a growth inhibitor isolated from African green monkey (BSC-1) cells¹⁸ and has many properties in common with the tumour inhibitory factor TIF-1 (ref. 19). Whether TGF- β acts to stimulate or inhibit the growth of a particular cell type seems to depend on many variables, such as the physiological condition of the cell and the presence of additional growth factors.

Here we report the amino-acid sequence of human TGF- β determined from direct protein sequencing and cDNA cloning. The nucleotide sequence of the TGF- β cDNA reveals that the 112-amino-acid-long TGF- β monomer is initially synthesized as part of a precursor polypeptide. Northern hybridization analysis demonstrates that the TGF- β mRNA is present in normal cells and in many types of tumour cells.

Purification and sequence analysis

A purification scheme has been developed by Assoian *et al.*¹¹ which yields TGF- β from human blood platelets, a major site of TGF- β storage. This procedure was modified and used to obtain human TGF- β with a purity of >95% for subsequent sequence analysis (Fig. 1 legend). In agreement with previous work¹¹ the non-reduced TGF- β migrated as an M_r 25,000 protein in a SDS polyacrylamide gel, whereas reduction with β -mercaptoethanol converted it into an M_r 12,500 species. This suggests that TGF- β consists of two M_r 12,000 polypeptide

chains linked by intermolecular disulphide bridges. The purified TGF- β was reduced, alkylated and subjected to amino-terminal sequence analysis. The amino-acid sequences of several peptides obtained after clostripain digestion of TGF- β were also determined. These analyses yielded a 60-residue long contiguous sequence starting from the amino terminus (Fig. 1a) as well as several shorter peptide sequences (data not shown). The NH₂-terminus of the human TGF- β is identical to the previously determined NH₂-terminal sequence of bovine TGF- β ⁸. The fact that no heterogeneity was seen during the NH₂-terminal sequence analysis, and that all TGF- β sequences obtained could be accounted for by an M_r 12,500 polypeptide, provide additional evidence for the homodimeric nature of this protein.

Isolation of TGF- β cDNAs

The approach we followed for the identification of the nucleotide sequence encoding TGF- β was similar to the strategy used previously for TGF- α ⁵. Long oligonucleotides designed on the basis of the partial protein sequence were used as hybridization probes for the identification of a TGF- β exon in a human genomic DNA library, which was then used as a probe for the isolation of TGF- β cDNAs.

Two 44-mer deoxyoligonucleotides, β LP1 and β LP2, complementary to sequences encoding amino acids 3–17 and 30–44, respectively, were chemically synthesized (Fig. 1a). The choice of these nucleotide sequences was based on previously described considerations⁵. In addition, 16 14-mers were synthesized which are complementary to all possible codons for amino acids 13–16 (Fig. 1a). A human genomic DNA library²⁰ was screened under low stringency hybridization conditions using ³²P-labelled β LP1 as probe. One of the 58 hybridizing phage, β LP58, also hybridized with the 44-mer β LP2 and with the pool of 14-mers. Nucleotide sequence analysis of this clone revealed the presence of a TGF- β exon encoding amino acids 10–60 of TGF- β . Only about half of the sequence corresponding to the β BP1 oligonucleotide is present. Nevertheless, the probe hybridized because of the correct assignment of 23 of the 24 nucleotides in the portion of the probe corresponding to the exon sequence (data not shown).

To obtain the entire TGF- β coding sequence, this exon was used as a probe to screen a λ gt10-based cDNA library derived from human term placenta mRNA. The screening of ~750,000 oligo(dT) primed cDNA clones resulted in the isolation of one TGF- β cDNA (λ BP1) of ~1,050 base pairs (bp). The previously determined partial TGF- β sequence established the reading frame and revealed the sequence encoding the complete TGF- β polypeptide, followed by a stop codon 20 bp from the 3' end of the cDNA.

Northern hybridization^{21,22} using the λ BP1 cDNA insert as probe showed that TGF- β mRNA of ~2.5 kilobases (kb) is present in the A172 glioblastoma and HT1080 fibrosarcoma cell

b

5' 3'

0 500 1000 1500 2000 2500 bp

λ BC5.7b

λ BC4.33

λ BC4.37

λ BC3.19

λ BC4.10

λ BC3.32

λ BC1

$\beta\lambda$ 58

TGF- β

Methods. TGF- β was purified from human platelets essentially as described previously¹¹ with an additional chromatography step over a C18 (Synchropak) HPLC column in 0.1% trifluoroacetic acid eluted with a 20–50% acetonitrile gradient. TGF- β was dialysed into 8 M urea and reduced by incubation in 0.1 M Tris-HCl (pH 8.5), 10 mM dithiothreitol, 8 M urea. Subsequent alkylation took place in the presence of 50 mM iodoacetate at room temperature in the dark. A fraction of the reduced and alkylated TGF- β was digested with clostripain⁴¹ and the peptides generated were purified by reverse-phase HPLC chromatography. Sequence determination took place using either an extensively modified Beckman 890C spinning cup sequencer⁴² or a vapour phase sequencer as described by Hewick *et al.*⁴³ (Applied Biosystems model 470A), with amino-acid derivative identification by reversed-phase HPLC on a Rainin Microsorb C-8 column. The oligonucleotides were synthesized using the phosphotriester⁴⁴ or the phosphite⁴⁵ method. The cDNA was prepared⁴⁶ from polyadenylated mRNA by priming with poly(dT)_{12–18} or the deoxyligonucleotide ACACGGGT-CAGGTAC (complementary to nucleotides 1,271–1,287). The double-stranded cDNA was treated with nuclease S₁ (Miles Laboratories) followed by subcloned into *Eco*RI-cleaved λ gt10 as described⁴⁷, except that asymmetric treatment. The recombinant phage were plated on *E. coli* C hybridized with ³²P-labelled⁵⁰ specific restriction fragments at 42°C in pyrophosphate, 5 $\times$ Denhardt's, 50 μ g ml⁻¹ salmon-sperm DNA and hybridization conditions⁵¹ were used for ³²P-labelled synthetic deoxy fragments was determined by the dideoxyligonucleotide chain termination method. The cDNA library was constructed by ligating the cDNA isolated from a human placenta cDNA library using the genomic exon A172 glioblastoma cDNA library (kindly provided by A. Ullrich) lead fibrosarcoma cDNA library with the ³²P-labelled *Kpn*I/*Kpn*I and the up 4.33 and 4.37. Another similar library was screened with the λ BC4.33 for the isolation of λ BC5.7b.

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lines. Extensive screening of cDNA libraries prepared from both these cell lines and of an additional placenta cDNA library yielded many different TGF- β cDNAs. Most of these seemed to contain sequence inversions generated during cDNA preparation ('Belgian' clones²³). In addition, single base deletions and substitutions were found in some of the cDNAs. Because of the high incidence of cDNA artifacts, presumably resulting from specific features in the mRNA or cDNA structure, all parts of the sequence were confirmed by sequencing at least two different cDNAs. Alignment of different cDNA sequences (Fig. 1b) enabled us to establish 2 kb of sequence preceding the TGF- β termination codon (Fig. 1c).

None of the 70 TGF- β cDNAs isolated from different oligo(dT)-primed cDNA libraries contained more than a few nucleotides of 3' untranslated sequences. As introns are rarely found in 3' noncoding regions, the 3' untranslated sequence was determined using cloned genomic DNA. DNA sequence analysis of phage $\beta\lambda$ 58 revealed the presence of an exon encoding the carboxy-terminal part of TGF- β , followed by the stop codon and the 3' untranslated region (Fig. 1c). An AATAAA hexanucleotide sequence²⁴ was encountered 500 bp downstream from the termination codon, thus permitting an assignment of the putative polyadenylation site. The calculated size of TGF- β mRNA is in close agreement with the 2.5 kb length determined from the Northern hybridization experiments (Fig. 2).

Carboxy-terminus of TGF- β

The cDNA sequence establishes that TGF- β is synthesized as the C-terminal segment of a precursor polypeptide. As several proteins undergo post-translational removal of C-terminal sequences, we determined the actual C-terminal amino-acid sequence of TGF- β . The cDNA sequence predicted the presence of a single methionine near the C-terminus in TGF- β . The protein was reduced, alkylated and cleaved with cyanogen bromide (CNBr). Sequence analysis of the mixture of peptides resulting from the CNBr digestion revealed the C-terminal sequence predicted from the cDNA (data not shown), although it cannot be excluded that a fraction of the molecule had undergone C-terminal proteolysis.

Unmodified TGF- β was also treated with CNBr. Cleavage at the methionine residue resulted in the complete loss of biological activity, showing that at least part of this C-terminal octapeptide is needed for biological activity (data not shown).

mRNA in different cell types

The availability of a TGF- β cDNA allowed us to screen various cells for TGF- β mRNA (Fig. 2). TGF- β mRNA was detectable in all human tumour cell lines including Wilms Tumour, A172 (glioblastoma) and the carcinoma cell lines T24 (bladder carcinoma), A431 (Squamous epidermoid carcinoma), MCF-7 (mammary carcinoma) and KB (nasopharyngeal carcinoma). HT1080, a fibrosarcoma-derived cell line, which we had chosen as a source of mRNA for the cDNA cloning, contained relatively high levels of TGF- β mRNA. TGF- β mRNA was not only present in cell lines derived from solid tumours of meso-, endo- and ectoblastic origin, but was also detectable in tumour cell lines of hematopoietic origin, for example Daudi (Burkitt lymphoma B lymphoblast), Raji (Burkitt lymphoma B lymphoblast) and Molt-4 (T-cell leukaemia). The presence of TGF- β mRNA is not restricted to tumour cells, as it is clearly detectable in placenta and peripheral blood lymphocyte (PBL) mRNA. Strikingly, the level of TGF- β mRNA is significantly elevated after mitogenic stimulation of PBLs. TGF- β mRNA was not detectable in human liver, yet was present in the HEP-G2 hepatoma cell line. In all cases, the TGF- β mRNA migrated as a species with an apparent length of ~2.5 kb.

Discussion

Analysis of several overlapping cDNAs and gene fragments has led to the determination of a continuous sequence of 2,439 bp corresponding to the TGF- β precursor mRNA. An initiator ATG is located 842 nucleotides from the 5' end and establishes

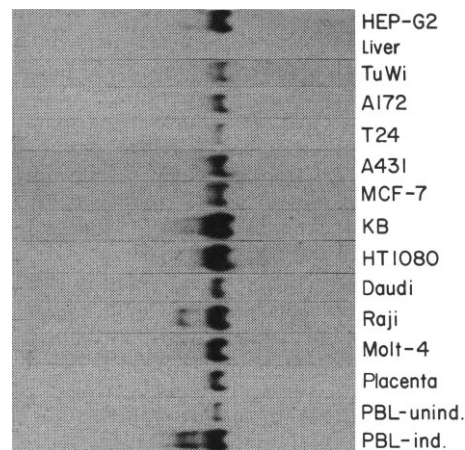


Fig. 2 Northern hybridization of mRNA from different sources with a TGF- β cDNA as probe. The cell source of the polyadenylated RNA is shown above each lane. The following cell lines were used: the hepatoma HEP-G2, Wilms tumour TuWi, glioblastoma A172, bladder carcinoma T24, squamous epidermoid carcinoma A431, mammary carcinoma MCF-7, nasopharyngeal carcinoma KB, fibrosarcoma HT1080, Burkitt lymphoma B lymphoblasts Daudi and Raji and T lymphoblast Molt-4. The cells growing in the monolayer were collected at confluency. The peripheral blood lymphocytes were prepared and mitogen-induced with staphylococcal enterotoxin B and phorbol myristate as described previously⁵⁴. RNA was prepared 24 h after induction. Comparison with the position of the 28S and 18S ribosomal RNA on the gel suggests a length of ~2.5 kb for the TGF- β mRNA. 4 μ g of polyadenylated mRNA was electrophoresed into a formaldehyde 1.2% agarose gel²¹ and blotted onto nitrocellulose filters²². The ³²P-labelled⁵⁰ *Eco*RI insert of λ BC1 was used as a probe under high stringency conditions (see Fig. 1 legend).

a coding sequence of 1,173 nucleotides, thus encoding a 391 amino-acid long polypeptide (Fig. 1b, c). Several areas in the cDNA sequence have an exceptionally high G-C content. In particular, the first 450 bp at the 5'-terminus has regions with >80% G-C content. The location of these G-C rich regions coincides with the areas in which the many cDNA cloning artifacts occurred and where partial length cDNAs were obtained. It is likely that the first ATG triplet located at position 842 (Fig. 1c) is the initiation codon as it is preceded in the same reading frame by several stop codons and it could initiate translation into a continuous sequence of a large polypeptide which comprises the TGF- β monomer. However, the sequence context at this ATG is not in good agreement with the proposed initiation codon consensus sequence G/ACCATGG²⁵. Furthermore, this ATG is localized in a very G-C rich area. A second ATG, found in the same reading frame at position 954, is in much better agreement with the initiation codon consensus sequence and is localized within a 40-nucleotide region of relatively low G-C content (~50%). This ATG may be more accessible to the ribosome, especially as it is localized within a large G-C region. Although it is more likely that translation starts at the first ATG, it is conceivable that the second ATG could also be used to initiate translation of a smaller TGF- β precursor.

The 5' untranslated region of the TGF- β mRNA is at least 841 nucleotides long and contains a 61-nucleotide-long sequence (positions 192-252) consisting almost exclusively of purines. The biological relevance of the exceptionally long 5' untranslated region of high G-C content is unknown, but it is similar to the structural organization of the mRNAs for *c-myc*²⁶ and insulin-like growth factor-2 (ref. 27). However, there is no striking sequence homology between these sequences. The first 120 bp of the TGF- β cDNA can theoretically be folded into hairpin loop structures with a calculated stability of -92 kcal (data not shown). It is tempting to suggest that the long 5' untranslated sequence and these hairpin loop structures could play a role in mRNA stability or in the regulation of transcription.

The stop codon at residue 2,016 is immediately followed by a remarkable, G-C-rich sequence of 75 nucleotides (Fig. 1c). This sequence consists of multiple repeats of CCGCC and ends with GGGGGC. The peculiar nature of this sequence is probably responsible for the fact that the 3' untranslated end of the mRNA could not be cloned as a cDNA sequence, perhaps because of the inability of the *Escherichia coli* DNA polymerase I to use this sequence as a template for the second strand cDNA synthesis. Similar repeat sequences have been found in the promoter regions of the genes for human dihydrofolate reductase²⁸, human adenosine deaminase²⁹ and Herpesvirus thymidine kinase³⁰. In the latter case, McKnight *et al.*³⁰ have shown that these structural elements are important in transcription efficiency. In addition, it has been shown that the promoter specific transcriptional factor Sp1 binds to such sequences in the simian virus 40 early promoter region and in a related monkey promoter³¹. In all these cases the G-C rich repeats are followed closely by the Goldberg-Hogness TATA sequence. In the case of TGF- β , however, these sequences are located in the 3' untranslated region of the gene, but, interestingly, are also followed by a TATA-like sequence. The hexanucleotide AATAAA, ~500 nucleotides downstream from the stop codon, probably functions as the TGF- β polyadenylation signal as this would agree with the size of TGF- β mRNA estimated from Northern hybridizations. Benoist *et al.*³² have proposed a consensus sequence TTCACTGC which immediately precedes the poly(A) tail. A similar sequence, TTCAGGCC, follows the AATAAA sequence in the TGF- β mRNA, providing further support for the assignment of the polyadenylation site at position 2,539 (Fig. 1c).

The TGF- β cDNA encodes a polypeptide of 391 amino acids, assuming that the first ATG is used for translational initiation. Comparison with the experimentally determined NH₂-terminus of TGF- β shows that the C-terminal 112 amino acids constitute the TGF- β sequence. The TGF- β monomer is cleaved from the precursor at the Arg-Arg dipeptide immediately preceding the TGF- β NH₂-terminus. Similar proteolytic cleavage sites have been found in several other polypeptide precursor sequences^{33,34}. Determination of the hydrophobicity profile³⁵ predicts that this Arg-Arg sequence is located within a hydrophilic region which would make it accessible to a trypsin-like protease. Whereas cleavage of the precursor gives rise to the mature TGF- β monomer, it is not known if other biologically active peptides are also released. The TGF- β precursor contains several pairs of basic residues (Fig. 1c) which could also undergo post-translational cleavage and give rise to separate polypeptide entities. However, the TGF- β polypeptide itself contains two Arg-Lys dipeptides which apparently are not cleaved. The TGF- β precursor contains three potential N-glycosylation sites³⁶, none of which are localized within the TGF- β polypeptide (Fig. 1c).

The 112 amino-acid sequence of TGF- β contains nine cysteines, whereas the rest of the precursor contains only three (positions 33, 224 and 226; Fig. 1c). There are no data either on the location of the disulphide bridges or on which cysteines are involved in the interchain disulphide linkage of the TGF- β dimer. Previous studies have shown that reduction of the TGF- β dimer of M_r 25,000 generates a single band of M_r 12,500 on denaturing polyacrylamide gel¹¹. Sequence analysis of the TGF- β amino-terminus and of the TGF- β peptides obtained after clostripain digestion strongly suggest that the TGF- β dimer consists of two identical polypeptides. This homodimeric nature is also supported by the presence of only a single hybridizing DNA fragment on Southern hybridization of human genomic DNA with a TGF- β exon probe (data not shown). Chou-Fasman analysis of the secondary structure shows³⁷ that the TGF- β polypeptide contains extensive β -sheet with little α -helicity. The region immediately preceding the basic dipeptide cleavage site is probably α -helical.

TGF- β is stored in relatively large quantities in platelets and is also found in association with mammalian cells. It is also released into the culture medium of these cells and, therefore,

is probably a secreted protein. The deduced polypeptide sequence of the 391-amino-acid-long TGF- β precursor shows a contiguous sequence of 16 hydrophobic residues (positions 8–23, Fig. 1c). This sequence may constitute the hydrophobic core³⁸ of an NH₂-terminal signal peptide involved in protein secretion. It is not known at which residue this cleavage by the signal peptidase could occur, but comparison with other known signal peptides shows an average signal peptide length of 20–30 amino acids³⁸. The mature TGF- β would subsequently be released from the precursor as a result of the cleavage by a dibasic peptidase, localized at the external side of the membrane³⁹. Translational initiation at the second ATG (position 954) would result in a 354-amino-acid-long precursor devoid of the putative signal peptide.

Most growth factors seem to be made only by specific cell types. PDGF is present in or released by platelets, endothelial cells, smooth muscle cells and various tumour cells. TGF- α is made by tumour cells of non-haematopoietic origin but has not been demonstrated to be synthesized by any normal adult cells. Previous studies have shown that TGF- β can be found in kidney⁸, placenta⁹ and platelets^{10,11} and is released by several 'normal' and transformed fibroblast cell lines. We examined several types of normal and transformed cells from different embryonic origin for the synthesis of TGF- β mRNA. Our results show that all cell types except adult liver examined contain TGF- β mRNA. The amount of TGF- β mRNA seems to correlate with the degree of mitotic activity, which may explain our inability to detect TGF- β mRNA in adult liver tissue. Furthermore, TGF- β mRNA levels are higher in mitogen-induced lymphocytes than in unstimulated lymphocytes. This correlation also seems to hold for the higher levels of TGF- β expression in transformed cells compared with their non-transformed counterparts⁴⁰.

It is clear that much additional work is needed to elucidate the role of TGF- β during cell division and propagation and in the establishment and maintenance of the malignant phenotype. The knowledge of the structure of TGF- β and its precursor and the use of TGF- β cDNAs as molecular probes will certainly contribute to this understanding.

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LETTERS TO NATURE

Evidence for H α emission associated with the HI bridge connecting the Small and Large Magellanic Clouds

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The bridge of matter connecting the Small Magellanic Cloud (SMC) with the Large Magellanic Cloud (LMC) was first detected thanks to radio observations, and detailed maps of this HI bridge are now available¹. Observations of the Magellanic system at H α wavelength reported by Johnson *et al.*² in 1982 seem to show a diffuse H α emission associated with the HI bridge. As they suggested, it is necessary to confirm this association by obtaining H α profiles in the direction of this bridge. We did this in February 1984 with the help of a scanning Perot-Fabry interferometer and a photon-counting system at the 1.5-m European Space Observatory (ESO) telescope. The choice of the area to be observed was based on recent observations³ with the Very Wide Field Camera (VWFC) on board Spacelab 1 (November 1983) showing an extension of the SMC towards the LMC at wavelengths of 1,930 and 1,650 Å. Detection of H α emission would check that the ultraviolet emission was emanating from the bridge connecting the two galaxies by showing that the cloud of hot stars giving rise to this ultraviolet emission was ionizing part of the HI bridge.

The patchy diffuse area detected by Courtès *et al.* with the VWFC³ is $\sim 1^\circ \times 2^\circ$ in size. Courtès suggested that we should obtain profiles of the H α emission line where ultraviolet maxima had been found ($\alpha = 1$ h 50 min, $\delta = -74^\circ 17'$ and $\alpha = 2$ h 10 min, $\delta = -74^\circ 34'$).

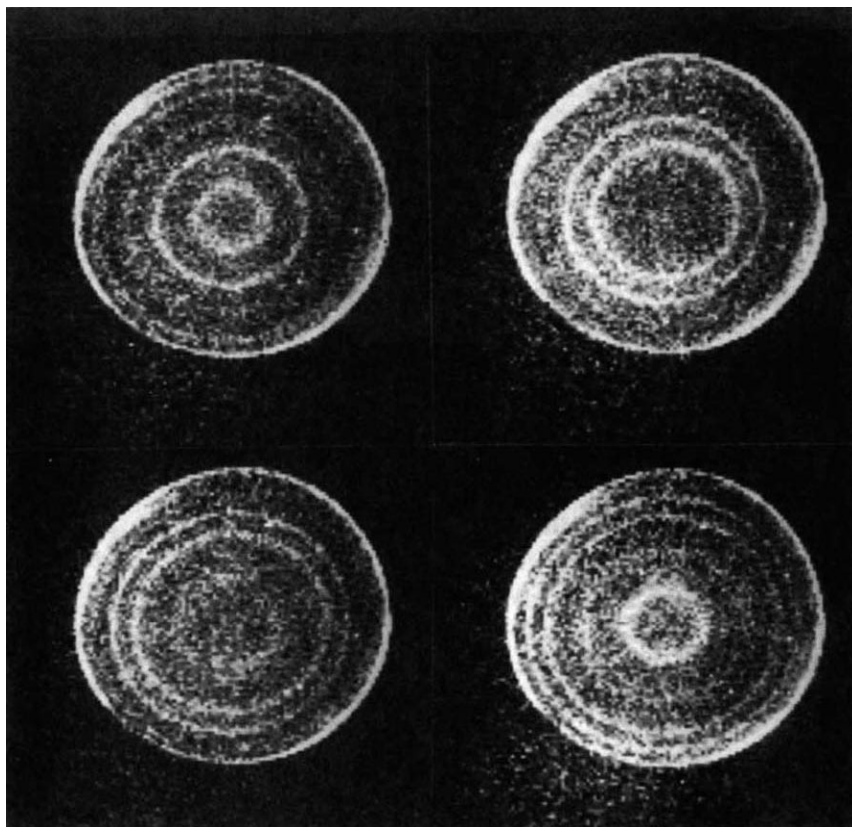
The instrument used for our observations has been described elsewhere⁴. We used a simpler version of the CIGALE experiment at the 1.5-m ESO telescope. The focal reducer was a smaller one, which was operated manually and which reduced the f :15 aperture ratio of the telescope to f :2.7.

The field of view is ~ 7 arc min in diameter and a 5-cm diameter filter was used (this filter was centred at 6,567 Å with a full width at half maximum FWHM of 10 Å). The centre of the first selected field is at $\alpha = 1$ h 50 min and $\delta = -74^\circ 17'$ and falls on chart 58 V of the Hodge and Wright⁵ photographic atlas of the SMC, approximately between reference points WG 5 and L114. The scanning Perot-Fabry interferometer used interference order 798 (at H α), giving 8.2 Å, or 376 km s⁻¹ for the free spectral range. The observed field has been scanned with 21 steps (each step, or channel, corresponding to a given spacing of the plates of the interferometer) ensuring complete coverage of the free spectral range. Figure 1 shows the real-time display of this observation on a TV monitor. Four channels are selected

(number 1, 6, 11 and 16, clockwise from the upper left corner) and displayed on a 256 times 256 pixel screen while the full data (photon addresses in a 512 times 512 pixel grid for each of the 21 successive channels) are recorded on a magnetic tape. Each channel was exposed for 20 s in each scanning cycle of 21×20 s = 420 s. Then the cycle was repeated 25 times. Thus the total exposure time was 500 s for each of the 21 channels, with the seeing conditions fairly well averaged for each channel over the total exposure by the scanning mode used. The analysis of the data shows that the brighter interference rings on Fig. 1 are due to geo-coronal H α emission (the innermost one on channel 1 of Fig. 1) and OH night-sky line at 6569 Å (the precise wavelength⁶ of which is $6,568.72 \text{ Å} \pm 0.02 \text{ Å}$). The faintest ring (the one closest to the centre in channel 11 of Fig. 1) is due to faint H α emission by the object observed in the ultraviolet by the VWFC on board Spacelab 1. The possibility that it was produced by any night-sky emission line has been ruled out because it was not detected on the second field which was observed during the same night ($\alpha = 2$ h 10 min, $\delta = -74^\circ 34'$) despite an exposure time three longer (but only unscanned this time). The H α emission line profile that has been detected is broader and fainter than those of the night-sky emissions. The width of the line is as much as 60 km s⁻¹ compared with ~ 20 km s⁻¹ for the geo-coronal H α and for OH (the resolution imposed by the Finesse of the Perot-Fabry interferometer). The detected emission is fairly uniform over all the observed field and we may estimate its intensity by comparison with the geo-coronal H α emission line. Comparing interference rings of same diameter for the two lines, we calculated the ratio of the number of counts and applied a small correction ($\sim 15\%$) to take into account the fact that each line is transmitted through the interference filter with a slightly different transmission factor, because of its narrow bandpass. As a result we found an average intensity ratio H α (object)/geo-coronal H α = 0.4 ± 0.1 . Similarly, we found OH(6,569 Å)/geo-coronal H α = 0.8 ± 0.1 . The problem with the intensity of the night-sky lines is that they are highly variable, depending on parameters such as the geographical location, date, the time of night, zenith angle, the phase of the solar cycle and so on. Noting the values given by Chamberlain⁷, (5-20 Rayleighs for the geo-coronal H α emission at the zenith) and if we consider 10 Rayleighs as a typical value, we conclude that the detected object had an intrinsic emission around 4 Rayleighs. Converting into more usual units this gives an intrinsic monochromatic brightness around $10^{-6} \text{ erg cm}^{-2} \text{ s}^{-1}$, sr⁻¹ or $10^{-9} \text{ J m}^{-2} \text{ s}^{-1} \text{ sr}^{-1}$. This result is in very good agreement with the Johnson *et al.*² observation of the SMC-LMC bridge; they found a maximum H α brightness around $2-5 \times 10^{-9} \text{ J m}^{-2} \text{ s}^{-1} \text{ sr}^{-1}$ in the bridge between the two clouds.

However, the most interesting result is the radial velocity measured from the Doppler shift of the H α emission of this object. Comparing the diameters of the observed interference rings with calibration rings produced by a H α lamp we first

Fig. 1 Real-time display of four scanning channels on a TV monitor (geometric distortions making the rings non circular are due to the monitor itself). Each channel has been exposed 500 s. 21 channels insured a complete coverage of the field (clockwise from upper left are channel 1, 6, 11 and 16). The brighter interference rings are due to geo-coronal $H\alpha$ emission (the innermost one on channel 1) and OH 6,569 Å night-sky line (the second one from centre on channel 1). The $H\alpha$ emission of the SMC-LMC bridge appears as a faint ring surrounding to two bright rings of airglow on channel 1. Then one can see the diameter of the interference rings increasing while the interferometer is scanning from channel 1 to 21. The $H\alpha$ emission of the bridge appears as a large faint ring together with a small central spot on channel 6, a small central ring on channel 11, and the second ring on channel 16 (where OH is the central ring). The bright circular edge on each image is due to the less selective transmission on the edge of the interference filter.



checked the wavelengths of the geo-coronal $H\alpha$ emission and the OH night-sky line. The wavelength accuracy found for both lines is better than $0.05 \text{ \AA}$ (2 km s^{-1} in radial velocity). The precision, indicated by the dispersion of the measured points, is about the same. The diameters of the $H\alpha$ interference rings from the object indicate an heliocentric radial velocity of $160 \text{ km s}^{-1} \pm 5 \text{ km s}^{-1}$ all over the observed field (note that the rough value given by us in Courtès *et al.*³ was underestimated). Our final result is in very good agreement with the average H I velocities found by McGee and Newton⁸ and by Mathewson (personal communication)⁹ in the same area. Furthermore, they generally found several components (3–4) with velocities ranging from 140 to 200 km s^{-1} and halfwidths around 20 km s^{-1} , which is consistent with the broadening observed for the $H\alpha$ emission. In some cases, our profile also appears to contain multiple velocity components but the signal-to-noise ratio remains too low to allow individual ones to be distinguished with certainty for their separations are near our spectral resolution.

It is clear, from the Doppler shift, that we are observing $H\alpha$ emission associated with the H I bridge between the SMC and LMC and that the large cloud of hotstars observed in ultraviolet light by the VWFC on board Spacelab 1 ionizes this bridge.

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The Sun's motion perpendicular to the galactic plane

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The period and amplitude of the Sun's motion perpendicular to the galactic plane are important parameters in some explanations of the terrestrial mass extinctions and cratering records^{1–5}. Here we have calculated the range of periods and vertical excursions that are consistent with the distributions of tracer stars in the Galaxy and have also evaluated the probable phase jitter in the solar period. We find acceptable half-periods for the vertical oscillation that range from 26 to 37 Myr (including the range of periods that have been inferred from the terrestrial records on mass extinctions and on cratering), maximum heights above the plane from 49 to 93 pc, and an average phase jitter per half-period of the order of 6–9%. The largest uncertainty in all these calculations is caused by the unknown distribution of the unseen stars that must be postulated to explain the distribution of observed stars^{6–7}. For all the models we consider, the most recent passage of the Sun through the galactic plane occurred in the past 3 Myr provided only that the present position of the Sun is between 0 and 20 pc above the plane. We extend the argument of Thaddeus and Chanan⁸ to show that the apparent periodicity in the mass extinction and cratering records cannot be caused by a population of objects (observed or unobserved) that contributes a major fraction of the total mass density at the solar vicinity.

We have calculated the time, t , that it takes the Sun to reach a height, z , above the galactic plane from the formula:

$$t = \int_0^z \frac{dx}{2(E - \varphi(x))^{1/2}} \quad (1)$$

where E is the total energy per unit mass of the Sun's vertical

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motion and φ is the potential energy per unit mass. The major uncertainty in evaluating equation (1) arises from the distribution of the unseen matter that is inferred to exist at the solar vicinity^{6,7}. We have bracketed the allowed solar motion by using the potential φ calculated for two extreme Galaxy models, labelled here as the ISM and the all-disk models. In the ISM model, the unseen material is squashed towards the galactic plane to the maximum extent allowed by the analysis of the distribution of F dwarfs and of K giants; the postulated distribution is similar to that observed for ordinary interstellar material (hence the name). In the all-disk model, the unseen material has the largest exponential scale height (0.7 kpc) above the plane that is consistent with the observed rotation velocity of the galaxy near the solar position. In the all disk model, all of the unobserved material near the Sun is by construction in a flattened disk; there is no appreciable amount of unobserved material in a spherical halo. We also consider a more conventional model, the proportional model, in which the distribution of unseen material with a given velocity dispersion is proportional to the distribution of observed material, with the same velocity dispersion, the constant of proportionality being independent of velocity dispersion. The proportional model has approximately equal amounts of unobserved and observed material. The particular parameters we use for these models are given in Table 5 of ref. 7, rows 1 (proportional model), 10 (ISM model), and 12 (all disk model). For all three models, the potential was derived by solving self-consistently the Poisson and the Vlasov equations with realistic models for the Galaxy (typically 15 observed components).

Table 1 shows our results for the calculated half-periods and maximum heights above the plane of the solar vertical motion. For each of the models, we also list the time since the Sun last passed through the galactic plane. The characteristics of the calculated solar motion depend on the vertical velocity, v_{present} , and height above the plane, z_{present} . We give results for the range of values that are consistent with the available observational results⁹⁻¹¹, that is $v_{\text{present}} = 6-8 \text{ km s}^{-1}$ and $z_{\text{present}} = 0-30 \text{ pc}$. The most likely values are considered to be 7 km s^{-1} and 4 pc , respectively, although some determinations^{12,13} suggest that the Sun is 20-30 pc above the plane. The Sun's position with respect to the galactic plane could be determined more accurately using modern surveys of H I and CO.

The half-periods we calculate are: $31.5 \pm 1 \text{ Myr}$ (proportional

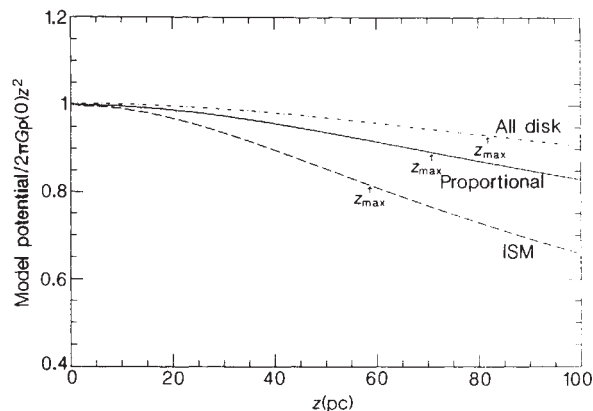


Fig. 1 The potential divided by $2\pi G\rho(0)z^2$ shown for the three numerical models.

model), $26.5 \pm 1 \text{ Myr}$ (ISM model), and $36 \pm 1 \text{ Myr}$ (all-disk model). The uncertainty due to the assumed current velocity and height above the plane is small compared with the difference between the values calculated with different distributions of the unseen material. For the most likely current parameters of the solar motion, the maximum height above the plane reached by the Sun, z_{max} , is: 70 pc (proportional model), 58 pc (ISM model), and 81 pc (all-disk model). The time since the last passage through the galactic plane is $< 3 \text{ Myr}$ in all the cases considered, provided that the Sun's current position is between 0 and 20 pc above the plane. The potential energy is currently much less than the kinetic energy for the solar vertical motion, so that the time since last passage is approximately the present height above the plane divided by the current velocity.

The problem of the solar vertical motion has usually been treated analytically by assuming^{8,14} that the potential for the vertical motion is quadratic in the height above the plane. In this approximation, the vertical motion is described by the equation for a simple harmonic oscillator. We show in Fig. 1 the potential, divided by $2\pi G\rho(0)z^2$, that was computed numerically for each of the galactic models we have discussed. Significant departures from the harmonic approximation are present beyond about 40 pc. Innanen *et al.*¹⁵ have evaluated numerically the solar motion in a simple galaxy model that includes departures from harmonicity.

How much of an error does the conventional assumption of a harmonic oscillator potential cause? The harmonic oscillator period differs from the exact half-period by 15% (4 Myr) for the ISM model, by 9% (3 Myr) for the proportional model, and by 6% (2 Myr) for the all-disk model. The error in the maximum height above the plane implied by the harmonic oscillator potential is, for the same three cases, 11, 6 and 4%.

The harmonic oscillator approximation can be used with relatively small errors in the computation of the fractional amount of time the Sun spends in any specified height range, once the period and maximum height above the plane have been calculated from the exact potential. We have evaluated numerically the fraction of time that the Sun spends in various height intervals using the potentials shown in Fig. 1 for the three illustrative galaxy models and have compared the results with the analytical formula¹⁴ that applies in the case of a harmonic oscillator. Adopting the exact values of the half-period, $P_{1/2}$ and of z_{max} , we find that the curve of elapsed times is described to an accuracy of 3% by the harmonic oscillator relation: $t(z)/P_{1/2} = \frac{1}{\pi} \arcsin(z/z_{\text{max}})$.

Some explanations⁵ attribute the apparent periodicities in the extinction data to phenomena occurring at the apex of the solar vertical motion. These explanations are unlikely to be correct because they would require an extinction event that lasts a time ΔT to occur all within $7 \text{ pc} \times (\Delta T/\text{Myr})$ of z_{max} .

How much phase jitter would one expect in the times at which the Sun crosses the galactic plane? We model the (unspecified) process that causes stars to have velocity dispersions that

Table 1 The Sun's vertical oscillation

Model	$\rho_{\text{total}}(z=0)$ ($M_{\odot}\text{pc}^{-3}$)	v_{present} (km s^{-1})	z_{present} (pc)	Half-period (Myr)	Last passage (Myr)	z_{max} (pc)
Proportional	0.21	7	0	31.4	0.0	70.0
		7	4	31.4	0.6	70.1
		7	10	31.4	1.4	70.9
		7	20	31.6	2.7	73.4
		7	30	31.8	3.9	77.4
		7	40	32.1	5.0	82.6
		8	4	32.0	0.5	81.2
		6	4	30.8	0.6	59.3
ISM	0.33	7	0	26.5	0.0	57.8
		7	4	26.5	0.6	58.0
		7	10	26.6	1.4	59.0
		7	20	26.9	2.7	62.5
		7	30	27.5	3.8	67.9
		7	40	28.1	4.8	74.6
		8	4	27.5	0.5	68.0
		6	4	25.6	0.6	48.6
All disk	0.15	7	0	36.1	0.0	81.1
		7	10	36.1	1.4	81.8
		7	20	36.2	2.7	83.9
		7	30	36.3	4.0	87.2
		7	40	36.4	5.2	91.6
		8	4	36.5	0.5	93.6
		6	4	35.7	0.6	69.0

increase with age by calculating simulated orbits in which a model star receives a vertical kick in velocity, δv , of random sign every half period. Solar type stars have¹⁶ a z -velocity dispersion of the order of 20 km s^{-1} after 5,000 Myr (the solar age), indicating that the Sun currently has an unusually small velocity perpendicular to the plane for a star of its age. To model what happens to typical solar-type stars, we adopt $\delta v \approx 20 \text{ km s}^{-1}/(\text{solar age}/P_{1/2})^{1/2} \approx 1.6 \text{ km s}^{-1}$. For anharmonic potentials like those shown in Fig. 1, the phase jitter per half-period, $\Delta\phi$, can be estimated analytically to be of the order of

$$\Delta\phi \approx \frac{0.2\delta v P_{1/2} n^{1/2}}{z_{\text{anh}}} \quad (2)$$

where z_{anh} represents approximately the anharmonicity of the potential in the relation $\phi(z) \propto z^2/[1+(z/z_{\text{anh}})]$ and n is the number of elapsed half-periods. In deriving equation (3), we have assumed that $z_{\text{max}} \ll z_{\text{anh}}$ and $\delta v \ll v_{\text{present}}$. For an elapsed time of 250 Myr, $\Delta\phi \approx 0.8 \text{ kms}^{-1} P_{1/2}/z_{\text{anh}}$. Values for z_{anh} can be estimated from Fig. 1; the half-periods, $P_{1/2}$, are listed in Table 1. For a harmonic potential, the phase jitter per half-period is zero if the velocity kick always occurs when the Sun crosses the galactic plane; the jitter decreases like $1/n^{1/2}$ if the kicks occur with equal probability between 0 and 40 pc.

We have carried out a series of Monte-Carlo simulations of the solar vertical motion including random kicks that are given with either a uniform probability between 0 and 40 pc or always in the plane. The results are shown in Table 2. The Monte-Carlo

Table 2 Expected phase jitter for solar period from vertical kicks in velocity

Model	Location of kick (pc)	$\Delta\phi$ (median)	Quartiles
Proportional	0.0	0.035	± 0.02
	0-40	0.045	± 0.03
ISM	0.0	0.07	± 0.035
	0-40	0.07	± 0.04
All disk	0.0	0.02	± 0.01
	0-40	0.03	± 0.02

simulations are in good agreement with the analytical estimate given in equation (3). The typical phase jitter after 250 Myr is in the range 0.03–0.07 per half-period, depending on the Galaxy potential that is adopted.

The vertical oscillation period is also changed by the epicyclic motion of the Sun in the plane of the galaxy. Let the half-amplitude of the epicyclic motion be X_{max} and let h be the exponential scale length of the disk density in the plane. The average period is only slightly affected by the epicyclic motion,

$$\text{Period}(X_{\text{max}}) \approx \text{Period}(X_{\text{max}}=0) \times \left[1 + \frac{X_{\text{max}}^2}{12 h^2} \right] \quad (3)$$

The average correction due to the epicyclic motion is $<1\%$ for typical values^{11,17} of h (3.5 kpc) and X_{max} (0.5 kpc). However, for times short compared with the period (≈ 160 Myr) of the solar epicyclic motion in the plane of the Galaxy, there is also a sinusoidal correction to the vertical oscillation period of amplitude $X_{\text{max}}/2h$. This correction could, in principle, be taken into account in making comparisons with observational data provided that the phase of the Sun in its epicyclic motion is not changed drastically during the time under consideration. On the other hand, the simplest (and most conservative) procedure is to ignore, when comparing with observations, the dependence of the period on the solar position in the galactic plane and to compute the total expected phase jitter by combining quadratically the previously computed jitter in the vertical motion (see Table 2) with an apparent phase jitter from the epicyclic motion in the plane. In this case, the expected r.m.s. phase jitter arising from the motion in the plane is $X_{\text{max}}/2^{3/2}h \approx 5\%$, comparable with the phase jitter caused by vertical kicks in velocity.

The combined calculated phase jitter per vertical half-period is in the range 6–9% for all the models we have considered. This calculated phase jitter is smaller than the observed jitter in the biological and cratering data.

Finally, we note that interactions with the unseen material cannot have induced the approximately periodic features that are inferred¹⁻⁴ from the extinction and cratering data. This conclusion follows from the work of Thaddeus and Chanan⁸, who showed that molecular clouds are not sufficiently concentrated towards the plane of the Galaxy to cause a strong preference for interactions at small heights. The unseen material cannot be more concentrated towards the plane than are molecular clouds. The maximum concentration of the unseen material towards the galactic plane that is consistent with the observations of F dwarfs and K giants is represented by the ISM model⁶⁻⁷, in which the unseen material has an exponential scale height which is like that of the molecular clouds (corresponding to a velocity dispersion of 4 km s^{-1}). Even in this extreme model, the maximum height above the plane reached by the Sun (49 pc) is about equal to the height (45 pc) at which the density of unseen material drops to e^{-1} of its value in the plane. The Sun encounters unseen material everywhere in its orbit, with only a moderate preference for interactions in the galactic plane.

All the observed components of the Galaxy that constitute major fractions of the mass density in the solar vicinity have scale heights that are comparable with or larger than the maximum heights, z_{max} , for the Sun listed in Table 1. If something associated with passages through the galactic plane is causing periodic mass extinctions and cratering, then whatever it is cannot constitute a large fraction of the mass density, either observed or unobserved.

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Increased concentration and vertical distribution of carbon dioxide in the stratosphere

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Although many measurements of the abundance of CO_2 in the troposphere have been made, knowledge of its stratospheric abundances and variability is sparse. Here we report mid-latitude vertical profiles of CO_2 , up to 35 km, measured in 1979, 1982 and 1984 by analysing cryogenically collected balloon samples supplemented by air samples taken aboard aircraft. CO_2 mixing ratios are not constant with altitude but rather decrease by $\sim 7 \text{ p.p.m.v.}$ (parts per 10^6 by volume) from the tropopause to the mid-stratosphere.

The growth rate of the atmospheric CO_2 abundance caused by anthropogenic emission, which varies between 1.0 and 1.5 p.p.m.v. yr^{-1} at ground level¹, is also observed at all stratospheric heights up to 35 km. The shape of the profiles suggests that excess CO_2 above 20 km enters the stratosphere through tropical upwelling rather than mid-latitude diffusion. The time lag of this height region with respect to the tropospheric CO_2 level is ~ 5 yr.

Cryogenic sampling provides a reliable technique for collecting large stratospheric whole air samples. With the Max-Planck Institut für Aeronomie (MPAE) balloon-borne cryogenic samplers using liquefied neon as a refrigerant, samples of 30 l STP can be collected in 500 cm^3 tubes, at 8 and 15 different heights, respectively. Subsequent gas-chromatography analyses allow for simultaneously determining the concentration of 23 stable source gases, such as H_2 , CH_4 , N_2O , CO and various halocarbons. Vertical profiles of these trace constituents, up to 35 km in altitude, have already been determined in this way²⁻⁴.

For CO_2 , the infrared absorption gas analysis provides a more precise analytical technique than gas chromatography; flask samples can be analysed with a total error of ± 0.2 p.p.m.v. (ref. 1). This method was applied to samples collected during 1977-79 balloon flights. The profiles obtained⁵ show CO_2 volume mixing ratios decreasing from a tropopause level to a mid-stratospheric level above 20 km, by ~ 7 p.p.m.v. this decrease taking place almost entirely within the 15-20 km altitude range.

It was found, however, that these first measurements, in particular those related to 1977 and 1978 balloon samples, had suffered from contamination problems because the sample aliquots had been stored for >1 yr. Moreover, the pressure of most samples was <5 bar; for such low pressures, repetitive analyses had shown that CO_2 concentrations can drift considerably. Thus, CO_2 data analysed from most 1977/78 samples had to be discarded. Samples from two flights in June and November 1979 were analysed with a delay of four months, but still several sample aliquots had a pressure <5 bar resulting in some scatter of the data. Figure 1 shows those of the 1979 CO_2 data analysed from stratospheric samples of >5 bar. Supplementary tropospheric CO_2 data analysed from samples collected aboard commercial airliners during June and November 1979 at mid-latitudes are also shown.

On 20 September 1982, 14 stratospheric samples were recovered from a balloon flight of the MPAE 15-tube sampler, at Aire-sur-l'Adour, France (44°N). Supplementary samples were collected on 23 September aboard Concorde between Paris and New York and aboard a Boeing 747 during the return flight on 25 September. Two years later, on 1 October 1984, a tandem flight of the 8-tube and the 15-tube cryogenic samplers, at Aire-sur-l'Adour, yielded 22 samples almost equally spaced between 10 and 35 km in altitude. Supplementary aircraft samples were collected on 20 September between Stockholm and Kiruna and on 4 October between Stockholm and Frankfurt.

All analyses of the 1982 and 1984 balloon samples were made without aliquoting, that is, the original sample air was transferred directly to the gas analyser. The absolute calibration was achieved with the aid of standard gases belonging to the Stockholm calibration gas system. These gases, consisting of mixtures of CO_2 in air and frequently being compared with the calibration system at Scripps Institution of Oceanography (SIO), are related to the '1974 adjusted index' calibration scale⁶. Corrections are needed for the carrier gas effect (SIO used CO_2 in N_2 mixtures) as well as for non-linearity and drift in the SIO calibration system. Because of uncertainties that still exist in the interpretation of calibration results we present our data in the 1974-adjusted index scale. We realize that true concentrations, within the range of CO_2 values reported here, are higher by $\sim 4.0 \pm 0.3$ p.p.m.v. With sample sizes ranging between 20 and 30 l STP, the CO_2 analyses were made within two months after the flights, when sample pressures ranged between 40 and 60 bar. Repetitive analyses made two months later showed no changes of the concentrations that exceeded the error limits of ± 0.2 p.p.m.v. The 1982 and 1984 stratospheric profiles of CO_2 displayed in Fig. 1 can therefore be considered with confidence. The same

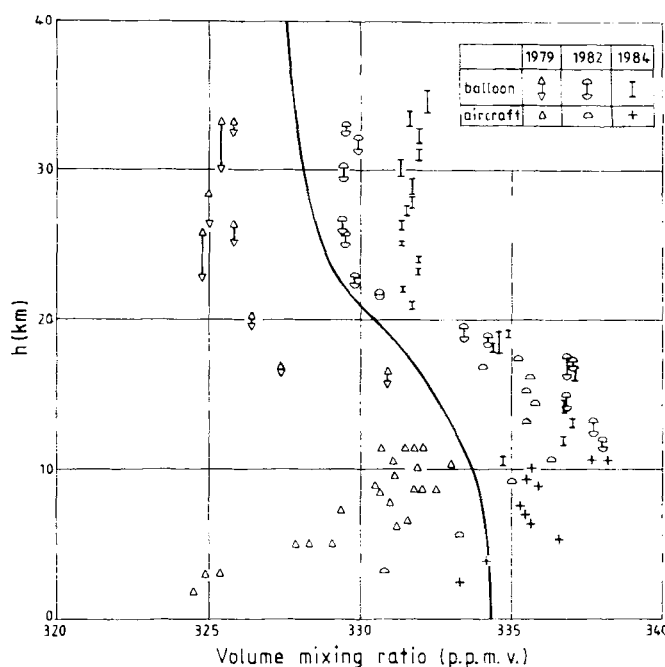


Fig. 1 Vertical distribution of CO_2 between the ground- and 35-km altitude as analysed by infrared absorption of whole air samples collected aboard balloon and aircraft platforms, for 1979, 1982 and 1984. The height range of the balloon samples is shown by the symbols. The modelled profile (solid line) computed by means of a one-dimensional time-dependent model corresponds to 1980 conditions.

holds for the aircraft samples analysed immediately after the flights.

The 1982 and 1984 CO_2 profiles confirm, in general, the existence of a decreasing CO_2 content with elevation. Even more important, they confirm that this decrease is confined to the height region between 15 and 20-22 km. The stratospheric CO_2 decrease begins ~ 5 km above the tropopause which, according to aerological soundings at nearby Bordeaux meteorological station, was located at 10, 11 and 12 km during the 1979, 1982 and 1984 balloon flights, respectively. Above 20-22 km, the CO_2 mixing ratio is almost constant with altitude; the 1984 profile even shows an indication of a slight increase above 30 km.

A striking feature of the CO_2 profiles presented here is the overall similarity of the stratospheric portions above 20 km. Obviously, the general increase of the tropospheric abundance of CO_2 resulting from the burning of fossil fuel is reflected by a stratospheric increase at a corresponding rate. Mid-stratospheric mixing ratios, as averaged over the height range above 22 km, are 325.4 ± 0.5 , 329.6 ± 0.2 , and 331.6 ± 0.3 p.p.m.v., for 1979, 1982 and 1984, respectively. Annual increase rates thus amount to 1.4 p.p.m.v. yr^{-1} between 1979 and 1982 and 1.0 p.p.m.v. yr^{-1} between 1982 and 1984 or an average of 1.2 p.p.m.v. yr^{-1} over the whole time range discussed here. These rates are comparable with those observed at tropospheric levels^{1,7}.

Annual means of tropospheric CO_2 as obtained from the Stockholm project, for the years discussed here, were found to be in excess of 6.8 ± 0.9 p.p.m.v. over the stratospheric mixing ratios reported here corresponding to a time lag of 5.2 ± 0.8 yrs. The tropospheric CO_2 profiles shown in Fig. 1 are representative of late summer/autumn conditions, when the cumulative uptake of CO_2 by the vegetation reaches its maximum, thus in the annual cycle a minimum is obtained in August/September. In late winter/spring, when CO_2 is returned to the atmosphere, a maximum occurs in April/May. This seasonal variation, having a total amplitude of ~ 7 p.p.m. in the northern upper troposphere, is almost undetectable within the lower stratosphere¹. However, a slight maximum of the order of ~ 1 p.p.m.v. above

the annual mean occurs in September. Thus, the results obtained from the lower stratosphere during autumn as shown in Fig. 1 may represent maximum rather than average conditions.

Compared with the fairly regular behaviour of mid-stratospheric profiles, the transition region below, between ~22 km and the tropopause, seems to reflect more complex features. The 1982 balloon profile, for instance, reveals exceptionally high CO₂ mixing ratios between 12 and 17 km that are comparable with or even higher than the corresponding 1984 data. As shown by the laminar structure of observed ozone profiles, transport in the lower stratosphere is largely achieved by large-scale quasi-horizontal advection. Near the tropopause, direct tropospheric/stratospheric mixing, often in connection with tropopause folding, may also be important. Either of these processes may be responsible for the high CO₂ values observed in 1982. Although some deviations between balloon and aircraft sample results exist, which are likely to result from different sampling locations, the CO₂ content below 16 km, in each of the 1982 and 1984 vertical distributions is nearly unchanged within this lower part of the stratosphere. There is a distinct change of the CO₂ mixing ratio from this layer towards the upper troposphere. This shift, as well as the rather uniform vertical distribution of CO₂ within the lower stratosphere, agrees with previous results of the Stockholm project⁸.

Our results show that stratospheric CO₂ concentrations increase with time at about the same rate as tropospheric abundances. The shape of the profiles, however, suggests that excess CO₂ from anthropogenic sources which are concentrated at northern mid-latitudes, does not simply propagate upwards there. A theoretical CO₂ profile computed for 1980 conditions by means of a time-dependent one-dimensional model, simulating vertical diffusion and following the observed growth of CO₂ at ground level³, clearly demonstrates that the observed profiles

cannot be reproduced by vertical diffusion alone. The fact that the mixing ratio gradient is confined to the height region between ~15 and 20 km and that CO₂ above that height is rather constant with altitude indicates that excess CO₂ must have entered the mid-stratosphere in a different way. We suggest that tropical upwelling is the mechanism by which the required vertical transport is probably achieved. It may carry CO₂ from the tropical troposphere to the middle and high tropical stratosphere, from where it spreads polewards. At all levels between 22 and 35 km, excess CO₂ obviously arrives with the same delay of ~5 yr with respect to the troposphere. The slight CO₂ increase above 30 km indicated by the 1984 profile suggests that the delay time for this upper region may even be shorter.

In this respect, measurements of stratospheric CO₂ profiles at tropical latitudes would be of great importance. Note that the existence of a shaped CO₂ profile as shown by our data may be relevant for satellite sounders that use the assumption of well-mixed CO₂ in the stratosphere to retrieve temperatures from infrared spectral features.

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Rate of sulphur dioxide emission from Erebus volcano, Antarctica, December 1983

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Mount Erebus is the principal source of volcanic emanations to the atmosphere in Antarctica. The rate of emission of SO₂ from Mt Erebus was determined to be 230 ± 90 tonnes day⁻¹ during an 11-day period in December 1983. This is a more accurate and much higher estimate than previous ones. Because of the very constant character of the volcano's activity from the earliest observations until September 1984, the emission rate is possibly representative of the past 20 yr. The new data are important for accurate assessment of volcanic input to the clean Antarctic atmosphere, and are a useful baseline for the changes in emission rates which will be measured now, after Erebus has begun a new energetic eruptive period.

Mt Erebus is the only active and the largest of three volcanoes which form the bulk of Ross Island, in the Ross Sea (77.55° S, 167.17° E). It is made up chiefly of anorthoclase phonolite lavas and has sustained an active lava lake, at least since 1972, and possibly for more than a century. Most of the observations of Erebus cite a persistent volcanic plume which emanates from its summit crater¹. Beginning in September 1984, Erebus has begun to exhibit much more explosive and prolific activity. The December 1983 period represents the low-level activity which

prevailed before 1984, and, therefore, represents a kind of baseline against which later observations, made during the more prolific activity, can be compared. Previously published estimates of the rate of emission of SO₂ by the Erebus plume have been 3 tonnes day⁻¹ in 1978 (ref. 2), and 35 tonnes day⁻¹ in 1980 (ref. 3). Interest in measurements of SO₂ and particle fluxes comes not only from volcanologists wishing to use such measurements to interpret the behaviour of the volcano, but also, particularly in the case of Erebus, from those trying to establish the sources of sulphate aerosol accumulations in the remote Antarctic environment⁴. Our objective was to make simultaneous and accurate airborne measurements of the gas and particle fluxes in the Mt Erebus plume. This is a report on the SO₂ measurements; we will discuss the particle data elsewhere.

To make measurements of the SO₂ fluxes, a correlation spectrometer (Barringer COSPEC-V) and an aircraft (C-130) survey method⁵ were used. The instrument is directed upwards and measures absorption of ultraviolet (290-300 nm) skylight, as the plane traverses under the plume, perpendicular to its dispersal axis. Such measurements give excellent estimates of the cross-section of the SO₂ burden, which must be multiplied by the wind speed to give the emission rate. On board the C-130 we were aided by continuous inertial navigation system readings of wind speeds (usually ~50 readings per traverse). Thus the largest source of error in such measurements was controlled very well compared with similar studies. Because of this, we believe our SO₂ flux estimates are of the highest quality. The previous estimates^{2,3} used much less direct methods and are more error-prone. Because the COSPEC measures only SO₂, the estimates obtained by this method are less than the total S flux of the volcano, because S is also produced as H₂S and as liquid and solid aerosol particles in volcanic plumes.

The general atmospheric conditions for sampling and measuring the plume on all four dates (9, 14, 17 and 19 December) were good, with clear visibility and light to moderate winds. Results of the individual SO₂ flux measurements and the measurement conditions of each of the traverses we made are

Table 1 SO₂ flux determinations, Erebus volcano, December 1983

SO ₂				
Traverse	Distance downwind (km)	Plume width (km)	Mean concentration (p.p.m. v.)	(tonnes per day)
a, 9 December 1983. Plume thickness, 300 m; winds SE at 5-7 knots				
1	3	6.6	0.30	160
2	6	13.5	0.26	360
3	6	14.4	0.26	440
4	6	14.2	0.26	440
5	3	6.0	0.26	170
6	3	9.3	0.18	150
1-6		$\mu \pm \sigma$		290 $\pm$ 140
b, 14 December 1983. Plume thickness, 100-200 m; winds W at 25-28 knots				
1	2	2.4	0.13	135
3	2	3.9	0.11	169
4	2	3.3	0.12	177
5	2	4.2	0.11	203
6	2	2.7	0.13	164
7	2	4.2	0.07	120
8	2	3.3	0.08	115
1, 3-8		$\mu \pm \sigma$		154 $\pm$ 32
c, 17 December 1983. Plume thickness, 300 m?; winds W at 8-10 knots				
1	3	8.7	0.10	242
2	3	8.1	0.13	295
3	3	9.0	0.10	306
4	3	8.7	0.08	221
5	3	10.2	0.07	240
6	3	10.5	0.11	414
7	3	10.2	0.10	302
1-7		$\mu \pm \sigma$		289 $\pm$ 65
d, 19 December 1983. Plume thickness, 150-200 m; winds WNW at 8-12 knots				
1	3	5.1	0.20	211
2	3	4.8	0.11	111
3	3	4.8	0.19	191
4	3	4.5	0.18	163
5	4	7.5	0.17	205
6	3	3.9	0.27	219
7	3	4.2	0.24	206
1-7		$\mu \pm \sigma$		195 $\pm$ 48

listed in Table 1. The wind conditions were variable and the geometry of the plume varied considerably throughout the period 9–19 December. Concentrations of SO₂ within the plume, based on the burden measurements and width and thickness data for the plumes, were in the range 0.07–0.30 p.p.m.v. (parts per 10⁶ by volume).

On the 9 December survey a fortunate event occurred. The plume, which from a distance had appeared white and billowy was momentarily darker in colour as we approached the volcano, 20 min before the survey. This darkening of the plume was probably due to a brief (1–2 min) strombolian explosion within the crater. Similar events have been described by Kyle *et al.*¹. Considering the timing of this brief explosion, the wind speed and the times of our COSPEC traverses, it is likely that the higher fluxes of traverses 2–4 (Table 1a) are due to this

explosion. It is also probable that this event affected our particle-size distributions, which were determined at a greater distance from the volcano after the COSPEC survey. The frequency of small explosions can be estimated from seismic disturbances which are correlated with them (J. Kienle, personal communication; based on reading the seismic records of the Erebus network at Scott Base) and seems to be about 1–3 per day. Because such explosions are infrequent, and probably last only a few minutes, we suggest that they do not increase the daily flux of SO₂ by more than a few per cent, even though the rate is several times higher while the explosions occur.

Table 2 summarizes the 4 days of SO₂ flux determinations at Erebus and demonstrates the overall average of 230 ± 90 tonnes day⁻¹. Considering our knowledge of the typical behaviour of Erebus, we believe this is a good estimate of the steady-state SO₂ emission of the volcano. Because we were fortunate to make measurements during one strombolian eruption, we can show that the occasional occurrence of such events does not affect the average flux significantly. The 1983 rate of emission for Erebus is lower than most volcanoes in an active eruptive state, which frequently have been estimated to emit >1,000 tonnes day⁻¹ (refs 6, 7).

Using the SO₂ emission rate it is possible to calculate the volume of the degassing magma chamber, by the approach of Rose *et al.*⁸. Electron microprobe analyses of glass inclusions in anorthoclase phenocrysts indicate a pre-eruptive sulphur content in the phonolitic magma of 440 p.p.m. (P.R.K., unpublished data). The sulphur content of whole rock samples ejected from the lava lake averages 260 p.p.m. (P.R.K., unpublished data). Assuming a magma density of 2.7 g cm⁻³ and 20 yr of degassing, a minimum magma volume of 1.7 km³ would account for the observed SO₂ emission rate. For 80 yr of degassing the volume of magma is 6.9 km³. Although Mt Erebus has been characterized by a large vapour plume throughout this century¹, the observations before the 1960s are particularly sparse and the calculated volume of 6.9 km³ appears to be unrealistically high. We prefer the 20-yr degassing volume of 1.7 km³. This is consistent with observations which indicate no significant change in the degassing and volcanic activity of Mt Erebus since the 1960s. Also, an aerial photograph taken in November 1963 indicates the existence of a small lava lake similar to that which existed from 1972 to 1982 (ref. 1).

The sulphur content of 440 p.p.m. in the Mt Erebus anorthoclase phonolite magma is low compared with reported concentrations of 800–2,800 p.p.m. S in basaltic and andesitic glass inclusions^{8–11}. The release of the sulphur from the magma, either in the form of H₂S and/or SO₂, will be a function of several variables, including oxygen fugacity¹². Estimates of sulphur and oxygen fugacity in the phonolite of log $f_{S_2} = -2.7$ and log $f_{O_2} = -12.2$ at 1,000 °C have been made of phonolite samples using the composition of pyrrhotite and other mineral phases¹³. In such conditions the fugacity of H₂S is likely to be high¹². One would therefore predict significant emission of H₂S in the Erebus plume. Analyses of gases within the phonolite glasses show a SO₂/H₂S ratio of ~2 (E. Bigelow and P.R.K., unpublished data). Very few accurate measurements of H₂S have been made in volcanic plumes⁷ and the rate of H₂S oxidation ultimately to sulphate particles is unknown for the Antarctic environment. However, H₂S must not be ignored when assessing the contribution of Mt Erebus to the atmospheric sulphur budget

Table 2 Summary of Table 1

Date (December)	Distance (km)	Width (km)	Concentration (p.p.m.v.)	Tonnes per day (means ± σ)	Winds (knots)	No. of traverses
9	3–6	6–14	0.18–0.30	290 ± 140	5–7, SE	6
14	2	2.4–3.9	0.07–0.13	150 ± 30	25–28, W	7
17	3	8–10.5	0.07–0.13	290 ± 65	8–10, W	7
19	3	3.9–7.5	0.11–0.27	200 ± 50	8–12, WNW	7
Overall $\mu \pm \sigma$				230 ± 90		27

for Antarctica. We are unable to quantify this contribution at present.

Our 1983 estimate of SO_2 emission from Mt Erebus is substantially higher than previous measurements. It is probable that Mt Erebus contributes significant amounts of sulphur to the Antarctic atmospheric sulphur budget. Radke³ suggested that Erebus contributed 32% of the sulphate to the budget. Delmas⁴, however, suggested that the effect of Erebus was small. Our measured SO_2 emission, excluding the possible contribution of H_2S , represents ~10% of the total Antarctic sulphur budget as estimated by Delmas⁴. Erebus is unlikely to influence the atmosphere over all of Antarctica, but its local effect may be significant. Because of the prevailing winds much of the sulphur is probably removed as aerosol over the Ross Sea, to the north of Erebus. However, as Delmas⁴ pointed out, the sulphur may be transported over large distances. Ultimately, it could circulate over the West and East Antarctic ice sheets. In addition, Erebus may contribute to the Antarctic stratosphere, especially during the winter months when the tropopause is significantly lower. A careful examination of the Antarctic sulphur budget, bearing in mind the Erebus contribution, is needed.

Because the activity of Erebus has been remarkably invariable in the period of human observations¹, we expect that the SO_2 emission rate we measured may represent a modern pre-1984 average for that volcano, which may be applied to the 1972–83

period and possibly for longer. We hesitate to conclude that the lower estimates of SO_2 flux in 1978 (ref. 2) and 1980 (ref. 3) show that the SO_2 emissions were increasing in late 1983 as a precursor of the increased activity, because the earlier estimates were made by very different methods. But the possibility that this does represent a precursive increase in SO_2 is consistent with experience at other volcanoes^{5,6}. We expect to continue SO_2 emission measurements throughout the current renewed activity to record the pattern and quantity of SO_2 release there.

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Direct measurement of air and precipitation particle motion by very high frequency Doppler radar

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Microwave meteorological Doppler radars are the most efficient tool for understanding the dynamical properties of precipitating systems in the troposphere¹. However, these radars do not directly observe ambient air motion, but rather the velocity of precipitation particles within a particular region. We have recently completed very high frequency (VHF) Doppler radar at Shigaraki, Japan (34.85° N, 136.10° E) which has enabled the three-dimensional motion of both air and precipitation particles to be observed simultaneously. In particular, it enables vertical air motion as well as precipitation fall speed to be directly observed. Doppler broadening of the precipitation echo reveals features which, when above the melting layer, are characteristic of snowflakes and, when below this layer, are characteristic of raindrops. Sensitive VHF (and, most likely, ultrahigh frequency, UHF) Doppler radars equipped with fast-beam steerability open new possibilities for the investigation of the dynamical properties of precipitation particles in close combination with ambient air motion.

MST (mesosphere–stratosphere–troposphere) radars are sensitive, high-power VHF/UHF Doppler radars that can detect clear-air (refractive-index) fluctuations or turbulence in the middle atmosphere (altitude range of 10–100 km^{2,3}). Received echoes are Doppler shifted because of radial motion of the clear air turbulence (CAT). The mean Doppler shifts acquired in three different directions provide three-dimensional velocity of CAT. This velocity is shown to be equal to the ambient air motion by comparison with measurements obtained by conventional techniques that use rawinsondes or meteorological

rockets^{2,4}. MST radars have rarely been used for the observation of tropospheric meteorological phenomena, with the exception of a few short-term studies of frontal zones⁵ and thunderstorms⁶.

Radar reflectivity of the inertia subrange turbulence in clear-air regions is dependent on the power law of $\lambda^{-1/3}$ (where λ is the radar wavelength). The reflectivity in the VHF band is observed to be within the range of 10^{-18} – 10^{-15} cm⁻¹ in the troposphere and lower stratosphere⁷. The power law yields a reflectivity of 5×10^{-18} – 5×10^{-15} cm⁻¹ in the microwave frequency band.

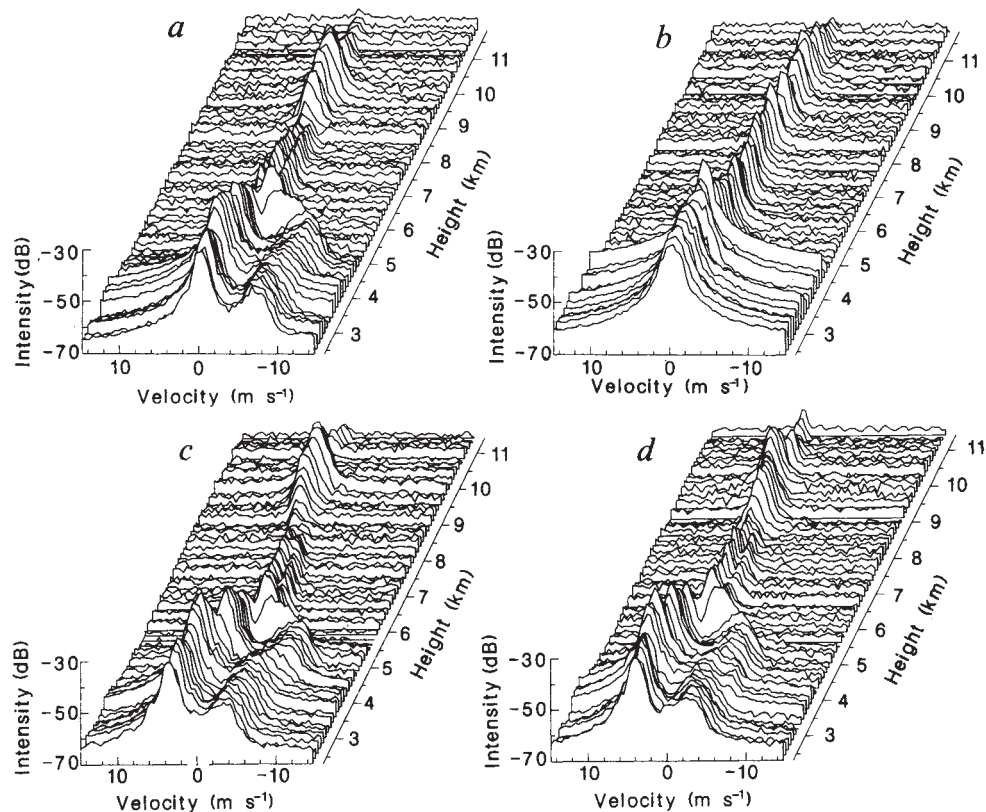
On the other hand, an estimate of the radar reflectivity for raindrops in the VHF band, for example, at 50 MHz, gives 10^{-19} – 10^{-16} cm⁻¹ for a precipitation rate of 3×10^{-1} – 3×10 mm h⁻¹, assuming the Marshall–Palmer drop-size distribution function (T. Yokoyama, personal communication). The reflectivity of the melting layer (or bright band) is ~50 times larger than that of raindrops. Thus, the reflectivity of precipitation particles at 50 MHz is within the range of 10^{-19} – 5×10^{-15} cm⁻¹, which is essentially the same range as that of CAT. This value is nine orders in magnitude smaller than that of microwave radars.

Because the reflectivities of both CAT and precipitation particles at 50 MHz are in the same range, sensitive 50-MHz MST radars are expected to detect simultaneously both types of echoes from precipitating air. This contrasts with the situation found in microwave radars, where precipitation echo is much more dominant than CAT echo. This is because the microwave reflectivity of precipitation particles is 4–12 order of magnitude larger than that of CAT.

The middle and upper atmosphere radar (MU radar) located at Shigaraki, Japan is a 46.5-MHz radar using an active-phased array system⁸. It is composed of 475 Yagi antennas and an equivalent number of solid-state power amplifiers (transmitter–receiver (TR) modules)⁹. Each Yagi antenna is driven by a TR-module with peak output power of 2.4 kW. The nominal peak and average radiation powers of the whole system are 1,000 and 50 kW, respectively. This system makes it possible to steer the antenna beam up to 30° from the zenith in each interpulse period.

Recently, we have observed precipitating air in the troposphere, using the fast beam steerability of the MU radar. We present the preliminary results of a short-term study which was successfully conducted on 22 August 1984. The full capabilities of the MU radar system were only partially employed. A nominal beam width of 4.0° and peak transmitted power of 760 kW were selected. Pulse width is 1.0 μ s, which corresponds to a range

Fig. 1 Doppler velocity spectra plotted against altitude obtained in the vertical direction by the MU radar, Shigaraki, Japan. The observational periods are: *a*, 0845–0851 JST (Japan Standard Time) and *b*, 0750–0756 JST on 22 August 1984. The ordinate is relative power in decibels with an arbitrary reference level. The abscissa is represented in terms of radial (vertical) Doppler velocity (Note that the sign is given in the reverse direction.) The positive and negative speeds correspond to departure from and approach to the MU radar, respectively. This usage is the opposite of the meteorological convention. *c*, *d*, Same as *a*, except *c* is northward and *d* is the eastward beam direction. The spectra are plotted against radial Doppler velocity.



resolution of 150 m. Echoes were sampled above 2.4 km from ground level (375 m above sea level) to obtain the delay required to protect the TR modules. The beam was steered during each interpulse period of 400 μ s sequentially to three different directions: zenith and 15° away from the zenith to the north and east. At an altitude of 5 km, the distance separating the off-vertical beams from the vertical beam was 1.3 km.

The bipolar video signal from each beam was sampled at 64 points spaced in the region from 2.4 to ~12 km aloft at 150-m intervals. 128 point complex fast Fourier transforms were calculated in real time to obtain Doppler velocity spectra for each 7.7-s period. The resulting power spectra were averaged for ~1 min before being written on magnetic tape.

Figure 1*a, b* shows typical altitude variations of the Doppler velocity spectra obtained in the vertical direction during periods (*a*) with and (*b*) without perceivable precipitation on the surface. Each spectrum is the average of a 6-min period. The surface rainfall rates during these periods were 1 and 0 mm h⁻¹, respectively, as observed by the Japan Meteorological Agency at their

facility in Kinose, 6.9 km north of the MU radar. The rainfall is classified as being of the weak stratiform type.

Of the spectral components illustrated in Fig. 1, the minor one with comparably large negative (downward) Doppler shift exists only when precipitation is observed (Fig. 1*a*), whereas the major one with near zero Doppler shift appears persistently irrespective of precipitation.

Figure 1*c, d* clearly illustrates the correspondence of these spectral components to those obtained in the northward and eastward beam directions. For the major spectral component, horizontal velocity is calculated by assuming the presence of a uniform velocity field within the region illuminated by the three beams (Fig. 2). The result is compared with the horizontal wind velocity measured by rawinsondes launched from Shionomisaki, ~150 km south of Shigaraki, and from Wajima, 250 km north. The radar-deduced horizontal velocity is consistent with the meteorological wind field, the difference between the three locations is in accord with that usually observed below 12 km (ref. 9). The vertical velocity shown in Fig. 2 is directly measured by the vertical beam.

Fig. 2 Altitude variation of vertical and horizontal components of air motion estimated from the major spectral component present in Fig. 1*a, c, d* (solid line). Positive vertical speed indicates updraft of air. Horizontal velocity is given in terms of wind speed and direction following the meteorological convention. The beams in the northward and eastward directions that are tilted 15° off from the zenith sample echoes at a range 100–300 m lower in altitude than that of the vertical beam. This altitude difference is taken into consideration when determining three-dimensional velocities by interpolating the vertical speed at the corresponding altitudes with a spline function. Comparisons are made with horizontal winds measured by rawinsondes launched from Shionomisaki, 150 km to the south of Shigaraki, and from Wajima, 250 km to the north, indicated by crosses and circles, respectively.

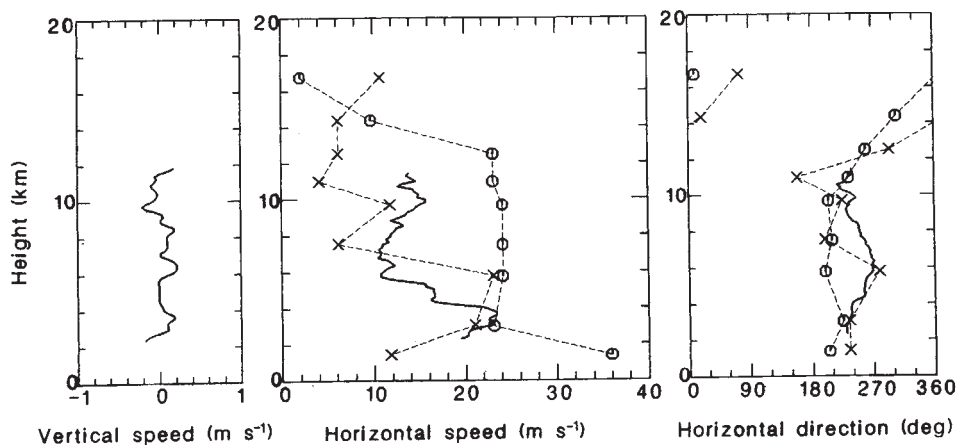
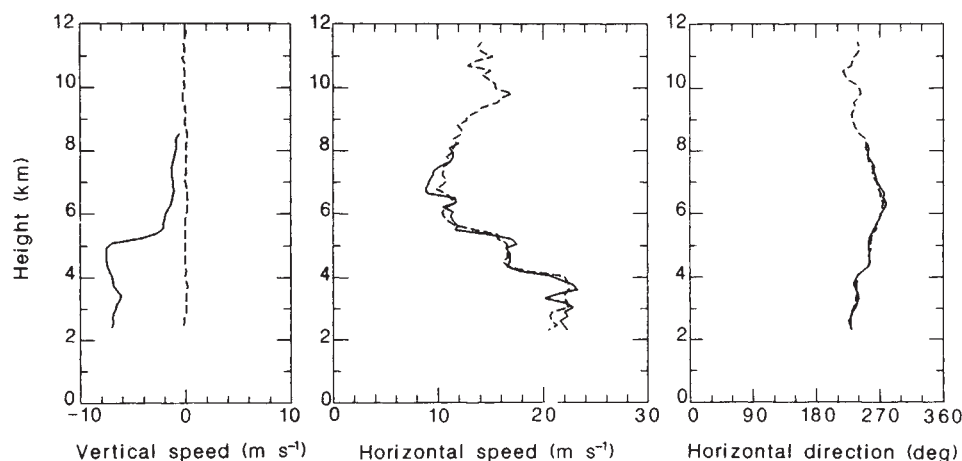


Fig. 3 Mean vertical (fall) speed and horizontal velocity of precipitation particles plotted against altitude estimated from the minor spectral component in Fig. 1a, c, d. Positive vertical speed indicates updraft. The ambient atmospheric wind velocity shown in Fig. 2 is also given by dashed lines for comparison. Precipitation particle motion is not given above 8.69 km, because the spectral component due to precipitation particles merges with that of ambient air origin above this altitude.



Below 5 km, the vertical speed of the minor spectral component is $\sim 7 \text{ m s}^{-1}$, whereas it is $< 2 \text{ m s}^{-1}$ above 6 km (Fig. 3). This change in the altitude range of 5–6 km is quite certain, because the minor spectral component clearly separates from the major one $\approx 8.5 \text{ km}$. It merges with the major component $\approx 9 \text{ km}$.

During periods when rainfall was perceived at the surface level, the melting layer was detected at 5.4 km by a dual frequency (C/Ku band) microwave radar located next to the MU radar¹⁰. Both spectral components of the 46.5-MHz radar echo are also enhanced at this altitude; the enhancement of CAT echo presumably results from a signature in the vertical temperature gradient in the melting layer.

The spectral width of the minor spectral component varies by more than three times near 5–6 km, ranging from 0.8 m s^{-1} above and 2.7 m s^{-1} below the melting layer. It is roughly constant elsewhere.

The above features concerned with vertical speed and spectral width are consistent with those of precipitation particles observed with meteorological Doppler radars^{1,11}. This would indicate that the minor spectral component of the MU radar echo originates from precipitation particles, that is, snowflakes above and raindrops below the melting layer. Note that, by using Doppler velocity spectra obtained by the vertical beam of the MU radar, drop-size distribution functions can be directly determined, obviating the need to make assumptions concerning vertical air motion. By and large, this type of direct measurement is not feasible using conventional meteorological radars.

Mean horizontal speed of precipitation particles is inferred in the same way as that of air (Fig. 3). The precipitation particles of the weak stratiform rain in the present example generally follow the ambient air motion. However, a detailed investigation of the differential motion would certainly be of interest.

In the present observation of a weak stratiform rain, vertical beam and two off-vertical beams with 15° off-zenith angles were used. This assumes that the wind field is uniform over the area containing the three beams, a region $\sim 2 \text{ km}$ at an altitude of 5 km. The assumption of wind field uniformity is necessary in monostatic wind vector measurements. However, it may be a source of error in convective storm environments. Depending on spatial scales of meteorological phenomena observed, a combination of three (or more) beams can be arbitrarily selected from the 1,657 directions within 30° off-zenith angle to which the MU radar can steer its beam. To provide significant three-dimensional motion, minimum beam separation can be reduced to beam width, or 350 m at an altitude of 5 km. This spatial resolution is sufficient to enable the observation of precipitating systems of a moderate size and larger.

Although the MU radar is capable of observing a substantial part of the deep and strong updrafts associated with frontal passages or convective storms which generally extend above an altitude of 10 km, it cannot provide the information concerning activities in the planetary boundary layer. Therefore, simultaneous observations with microwave meteorological radar

and/or other conventional instruments (such as the rawinsonde) will have an important supporting role in future investigations of the tropospheric meteorological phenomena which employ the MU radar.

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Indirect electrochemical degradation and dissolution of coal by means of persulphate

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The idea of electrochemical coal conversion has recently been revived^{1–7}. The mechanism of anodic coal oxidation has been shown to be indirect and that the electron transfer is mediated by $\text{Fe}^{3+}/\text{Fe}^{2+}$ leached out of the coal. Although the application of various mediator systems has been discussed in detail, all these systems suffer from either comparatively low reaction rates or high CO_2 current efficiencies. Caro's acid (H_2SO_5), obtained as a hydrolysis product during anodic persulphate formation is a powerful redox mediator system for the degradation of the macromolecular coal structure in mild conditions, providing a nearly complete dissolution of coal in sulphuric acid. We propose here that the high current efficiencies accompanied by low CO_2 evolution might open a route of potential interest for the electrochemical production of aromatic carboxylic acids from coal.

This redox mediator system has not previously been described, although there seems to be a similarity between Caro's acid and TFA (trifluoroperacetic acid) the application of which is described elsewhere^{8,9}. However, there are two significant differences. First, TFA destroys aromatic structures to such an extent that Deno proposed TFA-oxidation as a suitable tool for estimating the aliphatic carbon content in coal. The second difference is that TFA cannot be anodically regenerated from

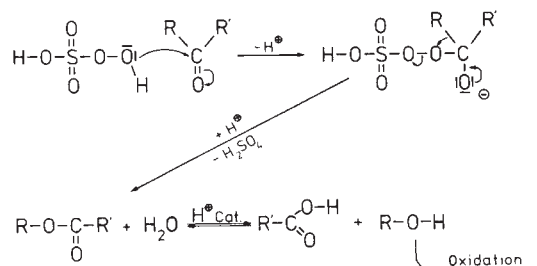
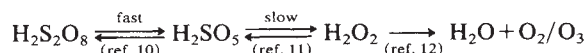


Fig. 1 Mechanism of oxidative coal cleavage by Baeyer-Villiger/Oxidation and consecutive saponification. R and R' represent the rest of the coal structure.

trifluoroacetic acid in conditions which are suitable for industrial application.

The special chemical and electrochemical features of the oxidant persulphuric acid have been described as follows: (1) Despite the high redox potential ($E^\circ = 2.05$ V), persulphate itself reacts, in general, very slowly. (2) In strongly acid solution, $\text{H}_2\text{S}_2\text{O}_8$ is hydrolysed depending on the reaction conditions in accordance with the following scheme



(3) The efficiency of anodic $\text{S}_2\text{O}_8^{2-}$ formation depends on the concentration of H_2SO_5 since the latter can depolarize the anode with formation of O_2 and H_2SO_4 (ref. 13). (4) Caro's acid (H_2SO_5) is a very strong and highly reactive redox mediator and oxidizes carbonyl groups giving rise to esters and lactones. The mechanism of this 'Baeyer-Villiger-oxidation' reaction, is given in Fig. 1. (All examples of the Baeyer-Villiger-oxidation up to 1953 are reviewed in ref. 14. Many of the compounds mentioned may serve as model substances for coal.)

Coal has a macromolecular structure containing condensed aromatic systems linked together by aliphatic bridges, the most abundant of which are methylene bridges. These units may be easily oxidized to carbonyl groups in the presence of $\text{S}_2\text{O}_8^{2-}$, SO_5^{2-} or H_2O_2 . A consecutive Baeyer-Villiger-reaction would result in the formation of esters which are supposed to undergo saponification in the strong acid media. By this sequence of reactions, C-C bonds connecting aromatic units in coal should be cleaved giving rise to the formation of low-molecular aromatic carboxylic acids. Because Caro's acid is consumed thereby, the current efficiency for anodic persulphate formation should be improved in the presence of coal according to point (3) above. These assumptions are confirmed by the experimental results which have been obtained in the system $\text{S}_2\text{O}_8^{2-}/\text{H}_2\text{SO}_4$ both with and without electrochemical regeneration of the oxidant.

If a given amount of coal (Westerholt-Kohle, West Germany; 2.4 g; <500 μm) is suspended in 300 cm^3 91 wt% sulphuric acid and stirred at 37 $^\circ\text{C}$ for 1 h no significant degradation of the coal takes place. If this experiment is repeated in the presence of $\text{Na}_2\text{S}_2\text{O}_8$, the electrolyte quickly turns brown and the coal quantity recoverable by filtration is decreased. This is shown in Fig. 2 for various amounts of $\text{Na}_2\text{S}_2\text{O}_8$ consecutively added to the suspension (curve a). The gas evolved during the reaction (curve c) is mainly O_2 formed by a parallel decomposition of Caro's acid. This gas contains only small amounts of CO_2 . The

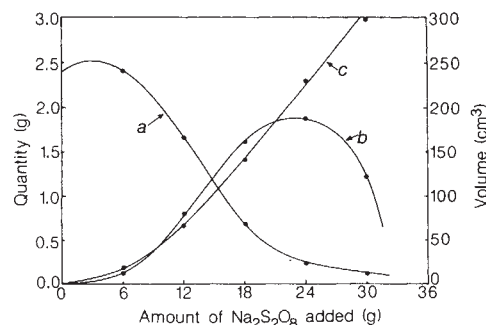


Fig. 2 Coal degradation as function of $\text{Na}_2\text{S}_2\text{O}_8$ consumption. a, Coal recoverable by filtration; b, precipitable acids; c, gas evolution.

losses of $\text{Na}_2\text{S}_2\text{O}_8$ due to gas formation were found to be <25% even at high degrees of coal oxidation.

The detached electrolytes from the experiments given in Fig. 2 were diluted with water in the ratio of 1:6. A deep-brown precipitate of carboxylic acids was collected, the amount of which is shown by curve b. The decrease of the precipitate yield at $\text{Na}_2\text{S}_2\text{O}_8$ consumption >24 g has to be attributed to consecutive oxidation of these intermediates. Thereby, a further breakdown can be achieved which finally results in the formation of low-molecular acids which are soluble and cannot be precipitated by dilution. Oxidative coal degradation follows the common sequence¹⁵:



But it is also known¹⁶⁻¹⁸ that the first step in this sequence is rate determining and cannot be obtained by mild oxidants such as H_2O_2 . Therefore, we were mainly interested in the formation of these intermediates, sometimes termed 'regenerated humic acids', because their consecutive conversion do not need the high redox potential of the persulphate system. In contrast with other oxidants, such as MnO_4^- , O_3 or HNO_3 a complete dissolution of coal can be achieved within a few hours using Caro's acid. Table 1 shows that there is a drastic change in the O/C-ratio between the intermediate acids and the native coal whereas, in spite of the reaction medium used, the sulphur content remains unchanged.

Infrared and nuclear magnetic resonance (NMR) spectral analysis data show that the aromatic basic structure of the coal is preserved within these acids. About 4-6 carboxylic groups per molecular unit were found whereas only a few aliphatic protons can be detected.

The experiments reported here were conducted at 37 $^\circ\text{C}$ with a H_2SO_4 concentration of 91 wt%. These conditions were found to be optimum with respect to reaction rate and $\text{Na}_2\text{S}_2\text{O}_8$ losses through gas evolution. At elevated temperatures in sulphuric acid of low concentration, the reaction mixture is decomposed to yield O_2 and O_3 with no significant dissolution of coal, whereas at higher H_2SO_4 concentrations and higher temperatures (~ 100 $^\circ\text{C}$) parallel SO_2 evolution was observed.

Kinetic investigations have shown that the reaction rate is strongly influenced by H_2SO_4 concentration and that in 3 M sulphuric acid no conversion of coal can be achieved. The same trend was found in HClO_4 . This fact is in accordance with the proposed reaction mechanism in which the real oxidizing agent is not the persulphate itself but Caro's acid the equilibrium concentration of which depends on the proton concentration¹¹.

Investigations^{13,19} have shown that a high current efficiency for electrochemical formation of persulphate can only be achieved in the following conditions: high current density of ~ 0.5 -1 A cm^{-2} ; low temperature in the range of ~ 10 -15 $^\circ\text{C}$; high SO_4^{2-} concentration; in conditions where the accumulation of H_2SO_5 in the electrolyte is avoided.

This situation is shown in Fig. 3a, where a current efficiency of $\sim 80\%$ is found at the beginning of the electrolysis which

Table 1 Ultimate analysis of the precipitable acids

	C	H	O	N	S	O/C	Average relative molecular mass
(g)	100	80.9	8.6	1.70	0.80	0.09	5,000-10,000
6	100	76.2	38.2	2.22	1.04	0.38	652
12	100	76.3	44.0	2.17	0.83	0.44	688
18	100	74.2	50.6	2.20	1.04	0.51	732
24	100	79.3	54.9	2.01	0.93	0.55	701
30	100	72.2	52.8	2.27	0.86	0.53	811

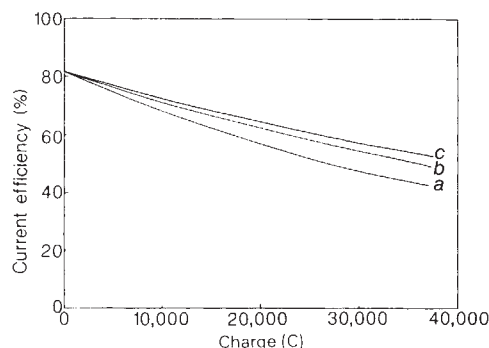


Fig. 3 Differential current efficiency of anodic persulphate formation with and without coal: a, no coal; b, with 7.36 g coal; c, with 14.72 g coal. $\text{H}_2\text{SO}_4 = 65\%$; temperature 10°C ; current 0.75 A; area of electrode 1 cm^2 ; volume of reactor, 460 cm^3 .

decreases with increasing accumulation of Caro's acid in the electrolyte. Three main effects are observed when the same experiment is repeated in the presence of coal.

First, neither the ash content nor the coal itself or its oxidation products reduce the current efficiency of the electrolysis. Second, no significant differences with respect to coal dissolution is found if electrochemically-regenerated persulphate were used. This can be shown by a further experiment. A total charge of 44,500 C has been applied corresponding to the formation of $0.134\text{ mol S}_2\text{O}_8^{2-}$ with an integral current efficiency of 58.1% determined by measuring the amount of oxygen produced. This electrochemically-formed $\text{S}_2\text{O}_8^{2-}$ dissolved 0.881 g of the 8.16 g of coal used in this experiment, corresponding to a relative coal dissolution of 10.8%. This means a relative persulphate consumption of $0.152\text{ mol S}_2\text{O}_8^{2-}\text{ g}^{-1}$ dissolved coal. For the chemical coal dissolution experiment (Fig. 2) at the same relative degree of coal conversion a persulphate consumption of $0.150\text{ mol S}_2\text{O}_8^{2-}\text{ g}^{-1}$ dissolved coal can be inferred which is in close agreement with the electrochemical experiment. Third, the decrease of current efficiency with time is not as steep as in pure H_2SO_4 , and depends on coal concentration.

The third observations were predicted on the basis of the reaction scheme outlined above, whereas the other observations could not be taken for granted.

The fact that there is still a decrease in current efficiency with increasing charge consumption, even in the presence of coal, has to be attributed to the low reaction rate caused by the low temperature needed in the electrochemical cell. Therefore, an outer cell process seems to be more appropriate where a coal slurry of high concentration is passing the electrochemical cell at low temperature levels and is oxidized in a chemical reactor at about $30\text{--}35^\circ\text{C}$. From the data given in Fig. 3 and from present investigations, a high constant current efficiency can be realized²⁰.

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Noble gases from oceanic island basalts do not require an undepleted mantle source

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Several lines of evidence based on relative rare earth abundances and on strontium, lead and neodymium isotopic geochemistry have led to the suggestion that the Earth's mantle may be divided roughly into a region depleted in volatile components, feeding the mid-ocean ridge basalts (MORB's), and a less depleted ('undepleted') region which feeds hotspots and thus oceanic island basalts (OIB's) such as Iceland and Hawaii. Models of mantle degassing and atmospheric evolution have been based on differences in argon and xenon isotope ratios measured in samples which supposedly trap rare gases from these two reservoirs¹⁻⁷. However, the ultra-mafic Ar and Xe that were first suggested as samples of the undepleted mantle were subject to air contamination and diffusion loss, so that the experimental results are not definitive⁸. New heavy rare gas data on volcanic glasses from Hawaii and Iceland are more directly comparable with the MORB glass data, and supposedly show the isotope pattern expected from the undepleted mantle^{2,5}. As I report here, however, absolute abundances and elemental ratios corrected for mass fractionation effects indicate that partial loss of the depleted mantle component plus addition of an atmospheric Ar and Xe component provide a better rationale for the isotopic differences in these heavy gases than does the postulated undepleted mantle component.

Table 1a summarizes the evidence cited. The trapped component seen in all oceanic glasses (Table 1a) has a $^3\text{He}/^4\text{He}$ ratio higher than atmospheric; as the source is shielded from any extraterrestrial influx, this must indicate a primordial component; the argument continues that the higher ratio seen in the supposedly undepleted glasses then indicates a more primitive source, that is, a more undepleted source.

This difference in ratios between the two mantle components could not have been quantitatively predicted *a priori*, because the undepleted mantle must contain not only higher abundances of the primordial gas (with respect to the depleted mantle), but also more uranium and therefore more radiogenic ^4He . The balance between the primordial ^3He and ^4He and the radiogenic ^4He is impossible to calculate because we do not know the fractionation factor between He and U in mantle processes, the fraction of the original mantle depleted in both the depleted and undepleted (actually merely less depleted) regions, or the time interval since the depletion process or processes occurred.

The $^{40}\text{Ar}/^{36}\text{Ar}$ ratio in the atmosphere is 295.5. The high ratio seen in the depleted samples results from potassium decay in the mantle and therefore is a firm fingerprint for a non-atmospheric origin. The nearly-atmospheric ratio in the undepleted samples has been interpreted as resulting from K-decay in a mantle region where the $\text{K}/^{36}\text{Ar}$ ratio is similar to that which has fed the atmosphere, and is much lower than that of the depleted mantle region because of a much higher primordial ^{36}Ar content⁷. However, the nearly-atmospheric $^{40}\text{Ar}/^{36}\text{Ar}$ ratio seen in the undepleted samples (Table 1a) could also be due to incomplete trapping of mantle Ar with unknown (perhaps depleted-type) isotopic ratios plus atmospheric contamination.

The higher than atmospheric $^{129}\text{Xe}/^{130}\text{Xe}$ ratios reported for (depleted) MORB glasses were interpreted as caused by the radioactive decay of ^{129}I after a significant fraction of the primordial Xe had been removed, implying that the depletion of the mantle took place early in the history of the Earth^{4,5}. This interpretation has been contested on various grounds (ref. 9 and R. Hart, personal communication), however, and conflicts with previous data from Hawaiian ultramafic nodules, where a slight anomaly was found in both the supposedly undepleted and

Table 1 Summary of rare gas ratios (a) and abundances (b) in oceanic glasses

Geographical location and type	Postulated mantle source	Ref.	a, Rare gas ratios			b, Rare gas abundances (cm ³ g ⁻¹ , STP)					
			³ He/ ⁴ He (×10 ⁻⁶)	⁴⁰ Ar/ ³⁶ Ar	¹²⁹ Xe/ ¹³⁰ Xe	³ He (×10 ⁻¹²)	⁴ He (×10 ⁻⁸)	³⁶ Ar (×10 ⁻¹⁰)	⁴⁰ Ar (×10 ⁻⁸)	¹³⁰ Xe (×10 ⁻¹⁴)	¹³⁶ Xe _f (×10 ⁻¹⁴)
MORB glasses	Depleted	5,12,15-18	12±2	500-25,000	648-690	35-450	1,400±1,000	0.3-7	150±100	6-30	0-2
Hawaii, Iceland glasses	Undepleted	2,5	17-50	350±50	648	4-40	20-170	1.5-12	0.3-35	4-30	0-1
Atmosphere			1.15	295.5	648						

depleted samples^{6,10,11}. The atmospheric Xe ratios in the OIBs may be evidence of a greater primordial/radiogenic ratio or, alternatively, of a greater atmospheric component.

I propose two arguments to the effect that the observed differences between the two groups of samples result from diffusion loss of mantle gases from and incorporation of atmospheric-type rare gases into the undepleted mantle samples, masking any possible Ar and Xe mantle signatures. First, consider the absolute abundance data for these samples shown in Table 1b. The undepleted mantle should have higher concentrations of all the rare gases than has the depleted system, yet the depleted samples show higher abundances of ³He, ⁴He, ⁴⁰Ar, and fissionogenic ¹³⁶Xe (¹³⁶Xe_f). (A very few MORBs have lower trapped rare gas abundances; the vast majority lie within the limits shown.) If the OIBs really represent undepleted mantle, it follows that they trapped the mantle gases less efficiently than did most MORBs. They were emplaced under shallower water depths, which may certainly explain the observed abundances; nevertheless, without arguing for any particular mechanism, the fact of lesser trapping efficiencies for the OIBs is inescapable if they are to be thought of as arising from the undepleted mantle. And yet these undepleted samples have generally higher abundances of ³⁶Ar, which is precisely the isotope most affected by atmospheric contamination. Thus, the absolute abundances indicate both incomplete trapping and atmospheric contamination, rather than support for the depleted/undepleted mantle concept.

This interpretation is supported by the second argument, which relies on elemental abundance ratios. Such values are notoriously unreliable as model foundations because they are easily influenced by mass fractionating mechanisms; however, some of the data sets have a built-in mass fractionation monitor, the ⁴He/¹³⁶Xe_f ratio¹². ⁴He is the radiogenic daughter of U and Th and their daughters, while ¹³⁶Xe is the fissionogenic daughter of ²³⁸U. (Allègre *et al.*⁵ erroneously claim ²⁴⁴Pu as the fissionogenic nuclide parent; see ref. 9 for a clarification of this point.) The ratio is constant over long periods of geological time, and other evidence indicates that accumulation of the radiogenic rare gases in these samples has indeed proceeded for time periods of the order of hundreds of millions of years⁹. Possible variations in the Th/U ratio introduce only a small variation. Thus, if the ratio found in a sample which has trapped mantle gases is higher than the production ratio of 4.2×10⁸ (±10%) (see ref. 12), it reveals the operation of positive mass fractionation (enrichment of the lighter gas), presumably during the mantle/crust emplacement process, while values lower than the production ratio indicate loss of the lighter gas (presumably through diffusion during or after eruption). If this radiogenic/fissionogenic ratio in a particular sample has been thus altered by mass fractionation, the comparable primordial ratio ³He/¹³⁰Xe must necessarily have been identically altered. Therefore, if a suite of samples has trapped rare gases from mantle source regions of identical He/Xe ratios, the measured ⁴He/¹³⁶Xe_f ratios will be proportional to the measured ³He/¹³⁰Xe ratios, regardless of whether any mass fractionation process has been operating on them:

$${}^3\text{He}/{}^{130}\text{Xe} = k_1 ({}^4\text{He}/{}^{136}\text{Xe}_f) \quad (1)$$

where k_1 is a constant. This is borne out by the MORB data of Table 2 (which includes all data sets for which the Xe isotope data are measured sufficiently precisely to allow an estimate of the fissionogenic component of ¹³⁶Xe): with a variation in

⁴He/¹³⁶Xe_f of a factor of 25 and more than an order of magnitude in absolute He and Xe abundances, k_1 remains constant to within ±50%. The surprise is that k_1 for the undepleted samples of Table 1b is identical. (The value for KK-29-10 is only an upper limit because only an upper limit to the ¹³⁶Xe_f can be calculated from the data.) This requires explanation. The ratio of trapped, mass-fractionated gases found in MORBs or elsewhere is related to that of the mantle source region by

$$({}^i\text{He}/{}^j\text{Xe})_{T,\alpha} = k({}^i\text{He}/{}^j\text{Xe})_{\alpha} \quad (2)$$

where i and j are mass numbers, k is a constant related to the i - j mass difference, α identifies the source region and T,α indicates the trapped component arising from source region α . Then, for a sample trapping rare gases from an undepleted source region ($\alpha = 0$),

$$({}^3\text{He}/{}^{130}\text{Xe})_{T,0} = k({}^3\text{He}/{}^{130}\text{Xe})_0 \quad (3)$$

$$({}^4\text{He}/{}^{136}\text{Xe}_f)_{T,0} = k({}^4\text{He}/{}^{136}\text{Xe}_f)_0 \quad (4)$$

leading to equation (1) where $k_1 = ({}^3\text{He}/{}^{130}\text{Xe})_0 / ({}^4\text{He}/{}^{136}\text{Xe}_f)_0$. The depleted mantle source region of the MORB will have lost more He than Xe in the depletion process (as is evidenced by MORBs showing ⁴He/¹³⁶Xe_f ratios higher than the production ratio), but the $({}^4\text{He}/{}^{136}\text{Xe}_f)_0$ ratio will soon be re-established through continued radiogenic production; the $({}^3\text{He}/{}^{130}\text{Xe})_0$ ratio, on the other hand, is permanently reduced. Therefore

$$({}^3\text{He}/{}^{130}\text{Xe})_{T,D} = k'_1 ({}^4\text{He}/{}^{136}\text{Xe}_f)_{T,D} \quad (5)$$

where D indicates the depleted mantle, and

$$\begin{aligned} k'_1 &= ({}^3\text{He}/{}^{130}\text{Xe})_D / ({}^4\text{He}/{}^{136}\text{Xe}_f)_D \\ &= ({}^3\text{H}/{}^{130}\text{Xe})_D / ({}^4\text{He}/{}^{136}\text{Xe}_f)_0 \\ &= k_1 ({}^3\text{He}/{}^{130}\text{Xe})_D / ({}^3\text{He}/{}^{130}\text{Xe})_0 \neq k_1 \end{aligned} \quad (6)$$

The ratio $({}^3\text{He}/{}^{130}\text{Xe})_D / ({}^3\text{He}/{}^{130}\text{Xe})_0$ is not unity because of mass fractionation during the depletion process, and therefore $k'_1 \neq k_1$. The value of the ratio can be estimated from the data shown here and in previous work. The reasoning is as follows.

Table 2 Rare gas elemental fractionation ratios

Sample	Ref.	$k_1 \times 10^{-5}$	$k_2 \times 10^{-5}$
'Undepleted' mantle			
Loihi Seamount			
KK-24-7	5	0.1	3.28
KK-29-10	5	<0.2	<7.0
Hualalai			
KK-9-14	5	0.13	1.28
MORB 'Depleted' mantle			
AI1 96-18-1	5	0.23	0.06
MD 23 Dr 04	5	0.073	0.04
ALV 981 R23	5	0.25	0.04
ALV 981 R26	5	0.076	0.04
ALV 892-2	5	0.084	0.098-0.21
AT-72	This work	0.090	0.065
AT-72	This work	0.083	0.060
AT-88	12	0.080	—
Average undepleted		0.12±0.02	2.30±1.40
Average depleted		0.13±0.07	0.06±0.02

Either the argon in the OIBs contains an atmospheric component or it does not. If it does, its isotopic composition is not representative of the undepleted mantle; it is only a lower limit to that composition, the exact value being dependent on the relative amounts of trapped mantle and atmospheric argon. If the argon in the OIBs contains no atmospheric component, we may use the measured argon concentrations in OIB and MORB samples as an estimate of the relative degassing that has taken place between the depleted and undepleted mantle, and therefore of the mass fractionation that must have occurred.

^{36}Ar in MORBs has been shown to be a mix of trapped mantle argon and atmospheric contamination^{9,13,14}; limits of trapped mantle ^{36}Ar are $\leq 1.5 \times 10^{-10} \text{ cm}^3 \text{ g}^{-1}$, STP. The highest ^{36}Ar content of OIBs is 12×10^{-10} (Table 1b). We assume for the moment that this represents argon trapped from the undepleted mantle rather than atmospheric contamination; it is a lower limit (compared with the MORB data), as it is clear from the He data that the OIBs have retained less of their mantle-inherited rare gases than have the MORBs. Then the basalts from the depleted mantle have trapped less than 10% of the argon trapped in basalts from the undepleted mantle, although their trapping efficiency is greater; the depletion factor must therefore be greater than an order of magnitude. Given that the mass fractionation occurring during the depletion is proportional to the square roots of the masses, it follows that the ratio $(^{3}\text{He}/^{130}\text{Xe})_{\text{D}}/(^{3}\text{He}/^{130}\text{Xe})_0$ remaining in the two mantles today must be less than 0.017 and therefore $k_1' \leq 0.017 k_1$. However, the data of Table 1b cannot be reconciled with this result; the single value of k_1 for both MORBs and OIBs flatly contradicts the expectation based on the concept of depleted and undepleted mantle regions outlined above. I suggest that the large enrichment of k_1 actually observed in the supposedly undepleted samples is a result of the $(^{3}\text{He}/^{130}\text{Xe})_0$ ratio in these samples being lessened by both loss of mantle ^3He and addition of atmospheric ^{130}Xe . This addition of atmospheric Xe is also responsible for the roughly atmospheric $^{129}\text{Xe}/^{130}\text{Xe}$ ratios in these samples.

The analysis can be extended to the Ar/Xe ratio:

$$^{36}\text{Ar}/^{130}\text{Xe} = k_2(^4\text{He}/^{136}\text{Xe}_t) \quad (7)$$

This time a distinct difference is seen between the two groups of samples (Table 2); the constant k_2 is much greater for the undepleted group, indicating either too much ^{36}Ar or too little ^{130}Xe . Since k_1 is identical for both groups, the simplest conclusion is that the samples interpreted as undepleted have an excess of ^{36}Ar . This excess of ^{36}Ar is the outstanding characteristic of samples which have undergone atmospheric contamination, as our atmosphere has a low $^3\text{He}/^{36}\text{Ar}$ ratio and a high $^{36}\text{Ar}/^{130}\text{Xe}$ ratio relative to the planetary primordial component.

The mode of incorporation of the atmospheric gases is unclear. The temperature release patterns of ref. 5 suggest that it is actually dissolved in the glass. If this is so, it may represent an atmospheric component injected into the magma source region rather than what is generally called 'atmospheric contamination'—surface adsorption following emplacement of the magma on the Earth's surface.

To conclude, the rare gas isotope ratio patterns of Table 1a, which have been offered as evidence of gases trapped from the undepleted mantle, are not convincing; two of the three (the $^{40}\text{Ar}/^{36}\text{Ar}$ and $^{129}\text{Xe}/^{130}\text{Xe}$ ratios) could equally well be explained by an atmospheric rather than an undepleted component. The data of Tables 1b and 2 cannot be explained by an undepleted mantle component but are perfectly explicable in terms of incomplete trapping and atmospheric admixture. The one data set that seems to indicate the existence of the undepleted mantle, the $^3\text{He}/^4\text{He}$ ratio, is also the one that would be least affected by the presence of an atmospheric component. It follows that modelling of mantle evolutionary histories and atmospheric formation which are based on Ar and Xe isotope differences between the depleted and undepleted mantle are not supported by such data as are now available.

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Thorium-232 in the eastern Caribbean Sea

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The radiogenic Th isotopes (^{234}Th , ^{230}Th and ^{228}Th) are produced *in situ* in the ocean from their dissolved progenitors (^{238}U , ^{234}U and ^{228}Ra , respectively). These parent–daughter pairs have extensively been used to study the rate and mechanism of chemical scavenging, a process that is believed to be important in controlling the distributions and concentrations of many trace metals, such as Be, Al, Cr, Mn, Fe, Co, Cu, Pb and the rare earths^{1–12}. However, it is difficult to make useful direct comparisons between the distributions of the radiogenic Th tracers and those of trace metals, because their input pathways to the ocean are very different. To provide a link, one needs to determine the water-column distribution of the dominant isotope ^{232}Th , which is non-radiogenic and is thus delivered to the ocean by the same fluvial and aeolian pathways as most trace metals. However, with the exception of the near-bottom measurements recently reported by Nozaki and Floribe¹³, little is known of the concentration level of ^{232}Th or its distribution pattern in the ocean. We report here the first profile showing the complete distribution of ^{232}Th in an oceanic water column from the Venezuelan Basin. The data were obtained by a sensitive neutron activation analysis method newly developed for this project. The profile shows relatively high concentrations in the upper 200 m with a maximum of $87 \text{ d.p.m.}/10^6 \text{ kg}$ near the base of the mixed layer. Average concentrations in deeper water ($2.7 \text{ d.p.m.}/10^6 \text{ kg}$) are uniform and atypically low compared with open-ocean deep waters, perhaps because of unusually rapid scavenging in the restricted basin. The source of the ^{232}Th in the surface waters is not known, but rivers appear to be more likely candidates than the atmosphere.

The samples were collected at $14^\circ 31' \text{ N}$, $66^\circ 8' \text{ W}$ during R/V *Knorr* Cruise 99–2 in November 1982. The seawater was filtered ($1 \mu\text{m}$ pore-size Nuclepore filters) and drained into 10-l acid-cleaned jerryjugs directly from the PVC Niskin samplers. Following collection, 12.5 cm^3 double quartz-distilled 8M HCl was added to each sample to bring the pH to ~ 2 . A full description of the clean sampling and filtration procedures used at sea is given elsewhere¹⁴.

Analysis in the shore-based laboratory began with additions of purified Fe^{3+} carrier and ^{230}Th spike to the acidified water. After several days for equilibration, ammonium hydroxide (ULTREX) was added to effect $\text{Fe}(\text{OH})_3$ precipitation. The

precipitates were collected by centrifugation and then dissolved in HCl. Separation and purification of Th-isotopes were done by anion and cation exchange and solvent extraction according to the method of C.-A.H.¹⁵ with minor modifications. The final source (in thenoyltrifluoroacetone/benzene) was mounted onto a high-purity (Puratronic) Al foil (2.5 cm diameter, 0.025 mm thickness). The Th recovery, based on ²³⁰Th, was determined with a proportional counter.

The sample foils, together with standard and blank foils, were packed in a polyethene vial and irradiated with a thermal neutron flux of 5×10^{13} neutron cm⁻² s⁻¹ for 6 h at the MIT Nuclear Reactor Laboratory. After removal from the reactor, the samples were allowed to cool for four days before the post-irradiation extraction of ²³³Pa (induced from ²³²Th).

The irradiated Al foils were dissolved in concentrated HCl and spiked with ²³¹Pa. The procedure for Pa (²³¹Pa and ²³³Pa) extraction was the same as that given in ref. 15, and the final source was made on a stainless steel disk. The Pa yield was determined by α -counting ²³¹Pa in a proportional counter. The ²³³Pa was determined by β -counting ²³³Pa in the same proportional counter. The purity of the Pa source was confirmed by the distinct 312-keV γ -radiation observed in a γ -spectrometer and by the decrease of β -activity being consistent with the half-life (27 days) of ²³³Pa. A complete description of the analytical procedures is given elsewhere¹⁶.

In dealing with the extremely low ²³²Th activities in the samples, we were aware of the potential for sample contamination and the importance of blank correction. Four sample blanks (reagents + Al foil) and three foil blanks (Al foil only) were prepared along with the samples and standards. Figure 1 shows, for these blanks, the relationship between the activity of ²³³Pa detected and the recovery of Pa in the post-irradiation chemical procedure. Two straight regression lines, one drawn for the sample blanks (SB1-4) and one for the foil blanks (FB1-3), are shown in Fig. 1. The offset of these two lines (that is, sample blank above foil blank) is a measure of the reagent blank, which turns out to be small compared with the foil blank. The good fit of the lines to the points indicates that ²³²Th on the Puratronic Al foil, although it exists at an easily detectable level, is evenly distributed in the material. The regression line for the sample blanks was used in making blank corrections for the samples and standards.

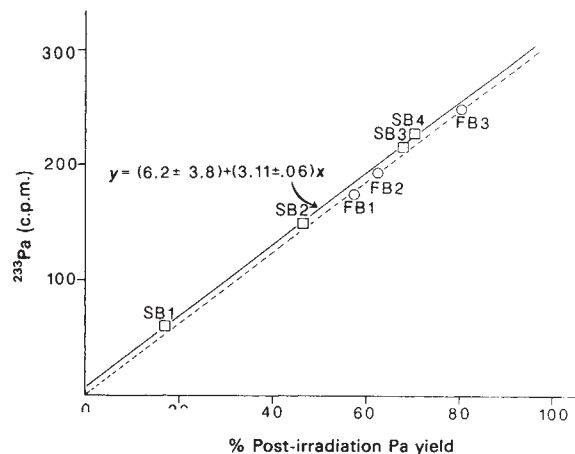


Fig. 1 The ²³³Pa/Pa yield relationship for the sample blanks (□) and Al-foil blanks (○). The slight offset of the two regression lines indicates that the foil blank dominates over the reagent blank.

Four ²³²Th standards, ranging from 0.01 d.p.m. to 2 d.p.m. (disintegrations per minute), were prepared from high purity ThO₂ (Lindsay Code 116, >99.9% purity) and run through the same procedures with the samples. A linear response resulted, as expected (Fig. 2); the regression coefficient is 1.24×10^6 (counts per minute, c.p.m. ²³³Pa/d.p.m. ²³²Th). The activities of ²³²Th in the samples were then calculated using the equation:

$$^{232}\text{Th (d.p.m. kg}^{-1}) = \frac{(\text{c.p.m. } ^{233}\text{Pa})_{\text{measured}} - (\text{c.p.m. } ^{233}\text{Pa})_{\text{blank}}}{(\% \text{ Th yield}/100)(\% \text{ Pa yield}/100) 1.24 \times 10^6 (\text{c.p.m.}/\text{d.p.m.}) \text{ sample wt (kg)}}$$

The results (Table 1) show that the sample blanks (reagents + foil) contribute 25–98% of the signals detected, resulting in uncertainties (by standard propagation of errors) of 2–80% for the final ²³²Th data.

The ²³²Th profile (Fig. 3) shows increasing concentrations with depth down to a maximum at 70 m, which coincides approximately with the base of the mixed layer defined by the temperature and salinity profiles. A weak suggestion of a

Table 1 Data used to calculate ²³²Th concentrations at the Caribbean Sea station (14°31' N, 66°8' W; water depth 4,886 m)

Sample no.*	Water depth (m)	Sample weight (kg)	% Th yield	% Pa yield	c.p.m. ²³³ Pa		²³² Th† (d.p.m./10 ⁶ kg)
					Measured	Corrected†	
601	10	10.6	70 ± 2	63 ± 2	272	70 ± 6	12.1 ± 1.1
602	40	10.8	80 ± 2	70 ± 2	642	418 ± 6	55.7 ± 1.6
603	70	10.8	83 ± 2	82 ± 1	1045	787 ± 3	87.4 ± 2.1
108	99	10.4	69 ± 2	73 ± 2	515	281 ± 6	43.3 ± 1.6
604	150	8.7	79 ± 2	80 ± 2	427	172 ± 6	25.2 ± 0.9
109	198	11.0	92 ± 1	90 ± 1	510	224 ± 3	19.8 ± 0.3
111	397	10.1	94 ± 1	55 ± 1	193	16 ± 3	2.5 ± 0.5
113	597	9.8	85 ± 2	63 ± 2	209	7 ± 6	1.1 ± 0.9
115	895	10.1	81 ± 2	74 ± 1	240	4 ± 3	0.5 ± 0.4
116	1,195	10.8	78 ± 2	59 ± 2	207	17 ± 7	2.8 ± 1.1
117	1,493	10.0	91 ± 1	83 ± 1	285	21 ± 3	2.2 ± 0.3
118	1,789	10.0	85 ± 2	94 ± 1	334	35 ± 4	3.5 ± 0.4
407	2,084	10.8	64 ± 2	92 ± 1	297	5 ± 3	0.6 ± 0.4
409	2,684	11.0	59 ± 2	49 ± 2	174	15 ± 7	3.8 ± 1.8
411	3,283	9.8	63 ± 2	76 ± 2	259	16 ± 7	2.8 ± 1.2
413	3,882	10.6	39 ± 2	79 ± 1	274	22 ± 3	5.4 ± 0.9
415	4,382	9.6	69 ± 2	80 ± 2	269	14 ± 6	2.1 ± 0.9
416	4,582	11.2	83 ± 1	92 ± 1	320	28 ± 3	2.6 ± 0.3
417	4,682	10.4	88 ± 1	63 ± 2	224	22 ± 6	3.1 ± 0.8
418	4,782	10.8	76 ± 1	61 ± 1	227	31 ± 3	5.0 ± 0.5

1 d.p.m. = 0.0167 Bq; 1 d.p.m. ²³²Th = 4.09 µg = 0.0176 µmol.

* First digit designates cast number; second and third designate bottle number.

† $^{233}\text{Pa}_{\text{corrected}} = ^{233}\text{Pa}_{\text{measured}} - ^{233}\text{Pa}_{\text{blank}}$, where $^{233}\text{Pa}_{\text{blank}}$ is estimated from Fig. 1.

‡ $^{232}\text{Th} = ^{233}\text{Pa}_{\text{corrected}} / [(\% \text{ Pa yield}/100)(\% \text{ Th yield}/100)(1.24)(\text{sample wt.})]$.

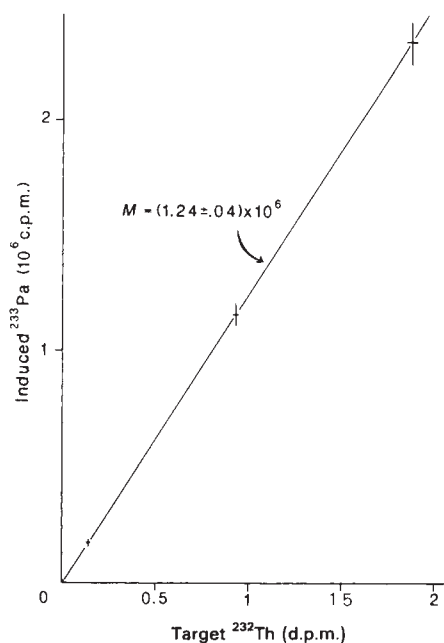


Fig. 2 Calibration line of ^{233}Pa detected (c.p.m.) versus ^{232}Th in the target (d.p.m.).

minimum is found at ~600–900 m. From 1,200 m to the bottom, the water column is very weakly stratified and there is no systematic trend of the ^{232}Th concentration with depth.

Many of the earlier reports of the ^{232}Th concentration in seawater acknowledged large uncertainties caused by the possibility of sample contamination and the large blank corrections that were necessary. Because all of our analyses of deeper samples (>200 m) showed consistently low concentrations (Table 1), we are confident that the much higher values we report for the upper 200 m accurately represent the concentrations in those samples. Our 10-m value is close to the upper limit of 17 d.p.m./ 10^6 kg for surface seawater set by Kaufman¹⁷ and later confirmed by Knauss *et al.*¹⁸. It is also within the range of 10–20 d.p.m./ 10^6 kg recently measured by Moore¹⁹ in surface seawater samples from the eastern Pacific. The one previously reported²⁰ value for surface Caribbean water (160 d.p.m./ 10^6 kg) is almost certainly too high.

The concentrations of ^{232}Th measured in the subsurface maximum are considerably higher than the upper limit for surface seawater given in earlier work. Whether these high concentrations are due to vertical particulate transport of ^{232}Th and its release at the depth of the maximum, or whether they are due to advective transport from a remote source, cannot be decided without additional data. During an earlier occupation of the same station, a ^{210}Pb maximum was found at nearly the same depth²¹.

Our deep-water values are considerably lower than most all others previously reported^{13,18–20,22,23}. The 14 samples from 397 to 4,782 m gave a mean concentration of 2.7 d.p.m./ 10^6 kg and a standard deviation of 1.4 d.p.m./ 10^6 kg. The results were close to our detection limit and could not be determined with very good precision. Improvement of precision at these very low concentrations would require larger samples or a reduction of the Al foil blank. The only value of comparably low magnitude that has been reported is the <2 d.p.m./ 10^6 kg determined by Anderson *et al.*²⁴ on a single sample from the deep North Pacific obtained from a moored adsorber. Recently, Nozaki and Horibe¹³, also using moored adsorbers, reported values ranging from 6.6 to 33.0 d.p.m./ 10^6 kg in the bottom 2,000 m at a 5,800-m station in the western North Pacific. Their values showed a regular decreasing trend with height above the bottom, suggesting a bottom source of ^{232}Th . We have not observed evidence for this in the Caribbean, where the deep water is well mixed.

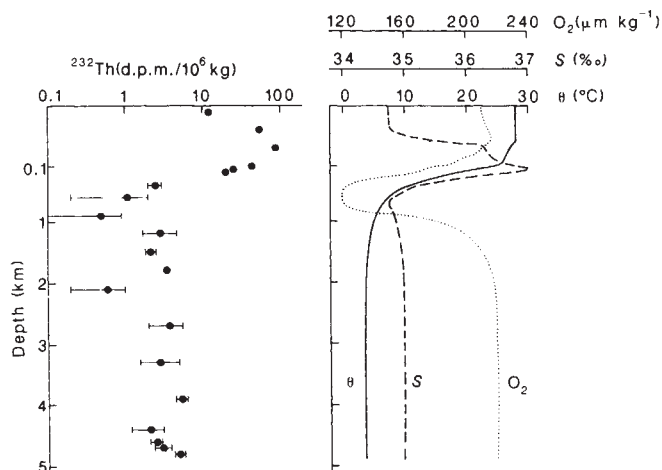


Fig. 3 Water-column ^{232}Th profile at the Caribbean Sea station ($14^{\circ}31' \text{ N}$, $66^{\circ}8' \text{ W}$). Also shown are the profiles of potential temperature (θ), salinity (S), and dissolved O_2 .

Although some of the earlier reported ^{232}Th data may be erroneously high because of contamination, it would be premature to reject them before further measurements are made. It may well be that ^{232}Th concentrations in the deep Caribbean are anomalously low by comparison with other regions. The overall pattern of the ^{232}Th distribution, with high concentrations near the surface and low concentrations at depth, is strongly suggestive of control by chemical scavenging in the deep water, and there is evidence that scavenging in the Venezuelan Basin is unusually rapid because of the enclosed nature of the basin and the proportionately large effect of uptake at the boundaries. The residence time of ^{210}Pb in the deep Venezuelan Basin was found to be only 20 yr (ref. 25), compared with values of ~50–200 yr more typical of open-ocean areas^{25,26}. Our unpublished ^{230}Th data obtained by large volume, *in situ* samplers, indicate a Th residence time of ~10 yr in the Venezuelan Basin, compared with open-ocean values of ~20–30 yr (refs 23, 27). Intense scavenging could maintain anomalously low concentrations of ^{232}Th .

The ^{232}Th concentration reported here for filtered seawater samples represents only a small fraction of the total. Our unpublished large volume results for the particulate matter gave an average of 22 d.p.m. $^{232}\text{Th}/10^6$ kg for nine samples collected below 200 m at the same station, or about 90% of the total ^{232}Th . This is in contrast to ^{230}Th , which is generated by decay of dissolved ^{234}U in the water column. Only ~30% of the total ^{230}Th occurs in the particulate form.

The new data reported here, showing the relatively high surface concentrations, raise the question of how dissolved ^{232}Th is supplied to the surface ocean—whether by leaching of dust delivered from the atmosphere or by river input. As discussed by Measures *et al.*²⁸, the question is a generally difficult one for trace metals, and with our limited data we can only advance a few tentative statements regarding the magnitude of the supply that is necessary. From our data, the inventory of dissolved ^{232}Th in the upper 200 m is 6.5 d.p.m. m^{-2} , and from studies of ^{228}Th , it is known that the residence time of dissolved Th in the upper ocean averages ~0.7 yr (ref. 29). Thus a supply rate of 9.3 d.p.m. $\text{m}^{-2} \text{ yr}^{-1}$ is required to maintain the inventory of ^{232}Th that we observe. Buat-Menard and Chesselet³⁰ estimated the flux of ^{232}Th in the form of atmospheric dust over the tropical North Atlantic to be 2.2 d.p.m. $\text{m}^{-2} \text{ yr}^{-1}$. Because only a small fraction of that flux would be leached on contact with surface seawater, this does not appear to be an adequate source. Moore³¹ reported a value of 0.024 d.p.m. $^{232}\text{Th} \text{ kg}^{-1}$ in centrifuged Amazon River water. Taking that value and the river flux to the Atlantic of 250 $\text{kg} \text{ m}^{-2} \text{ yr}^{-1}$ yields a ^{232}Th flux of 6 d.p.m. $\text{m}^{-2} \text{ yr}^{-1}$, not much lower than the required supply rate estimated above. Considering that the Caribbean is probably more affected

by river input than the average Atlantic surface water, river input may be an adequate source of ^{232}Th , although nothing is known about the estuarine behaviour of Th.

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Regressive events in the postnatal development of association projections in the visual cortex

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In newborn kittens, neurones in area 17 of the visual cortex projecting to area 18 are distributed in bands of uniform density across the superficial layers (laminae II, III and the upper part of IV) and the deep layers (V and VI). During weeks 2 and 3 postnatal, the cells of origin of this association pathway become mainly restricted to discrete, dense clusters, ~600 μm from centre to centre, in the upper layers, with intervening zones free of association cells^{1,2}, as in the adult cat^{3,4}. We have used retrogradely transported, long-lasting neuronal markers to investigate this developmental refinement of the pattern of cortico-cortical connections. The results, reported here, indicate that axonal retraction plays a significant part in the maturation of the clustered organization of superficial layer neurones projecting to area 18, but that cell death may also be a factor in the elimination of the inappropriate projection from the deep laminae.

Recent research has demonstrated that certain neurones in the mammalian cerebral cortex, early in development, have axonal connections to targets that would be inappropriate in the adult animal. For the long interhemispheric and cortico-spinal pathways, the postnatal elimination of these initially 'exuberant' projections seems to be achieved by axon retraction without death of the cells of origin^{5,6}. Neuronal death certainly occurs in many parts of the developing nervous system⁶, including during the early stages of formation of the cerebral cortex⁷, but it has not yet been shown to be involved in the elimination

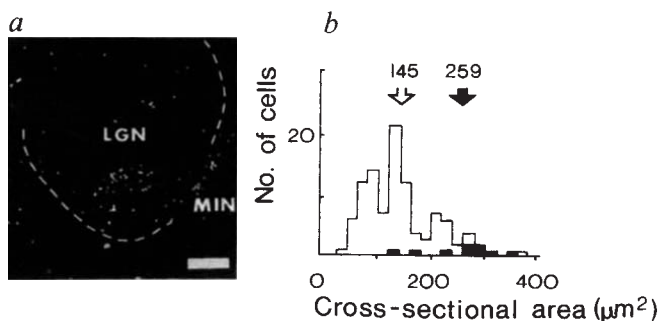


Fig. 1 Analysis of LGN cells retrogradely labelled following an injection of WGA-HRP into area 18 in a 4-day-old kitten. *a*, Dark-field illuminated coronal section through the thalamus, showing labelled cells lying in a central position in the LGN, as well as in the medial interlaminar nucleus (MIN). The lateral and ventral borders of the LGN are indicated by the broken line. Scale bar, 1 mm. *b*, Histogram of cross-sectional areas of a sample of cells from the middle of the region of uptake. Solid blocks represent labelled cells; mean values (in μm^2) are indicated for total (open arrow) and labelled (solid arrow) samples. Injections that produced labelling of relatively large, centrally placed cells in the LGN were considered to be confined to area 18.

of exuberant cortical projections. In the present study we examined the factors involved in development of the relatively short cortico-cortical projections from area 17 to area 18 in kittens.

Kittens were anaesthetized with halothane, fixed in a stereotaxic holder⁸ and axonally transported tracers were injected into area 18 of the visual cortex. Three animals, two aged 2 days and the third aged 2 months, were given a single 500 nl injection of a 2% solution of the fluorescent tracer fast blue (FB) via a pulsatile pressure system through a glass micropipette (tip diameter ~50 μm) into area 18 on the left side of the brain. These kittens then survived for 4 weeks before perfusion with a 4% solution of paraformaldehyde. In one of these animals the initial injection of FB at 2 days was followed at 1 month old, 24 h before perfusion, by a second injection of diaminidino yellow (DY; see ref. 9) into area 18 close to the original FB injection site, using identical methods. In a further four kittens (aged 1, 4, 10 and 20 days) area 18 was injected iontophoretically (repetitive positive current pulses of 3 μA for 2 h) with a 10% solution of horseradish peroxidase conjugated with wheat-germ agglutinin (WGA-HRP) dissolved in a solution of Tris(hydroxymethyl)methylamine; these animals were perfused either 8 or 24 h later with 1.25% glutaraldehyde and 1% paraformaldehyde.

Coronal sections 50 μm thick were cut through the visual cortex and lateral geniculate nucleus (LGN) on the side of the injection; material containing WGA-HRP was reacted using tetramethylbenzidine as the chromogen¹⁰ and counterstained with neutral red. Sections were examined using light or fluorescence microscopy and drawings were made of the positions of all labelled, or, where appropriate, double-labelled cells, within the cortex and LGN. For each animal a series of sections through the visual cortical areas was counterstained with cresyl violet and another was reacted to reveal cytochrome oxidase activity¹¹. We made high-power drawings of the outlines of all labelled cells (in which the nucleus and nucleolus were visible) within an area in the centre of retrograde labelling in the LGN, and the cross-sectional areas of these cells were calculated using a graphics tablet linked to a computer. The sizes of these labelled cells were compared with those of a sample of intermingled unlabelled Nissl-stained LGN neurones from within the same area.

The injection sites of FB and DY in area 18 were all ~1 mm wide but covered the entire depth of the cortex so as to expose axon terminals in all laminae to the tracer. Haloes of staining around the deposits of WGA-HRP were slightly larger and more variable in size (up to 2 mm wide), although the effective uptake sites of WGA-HRP¹⁰, estimated from the pattern of label in the

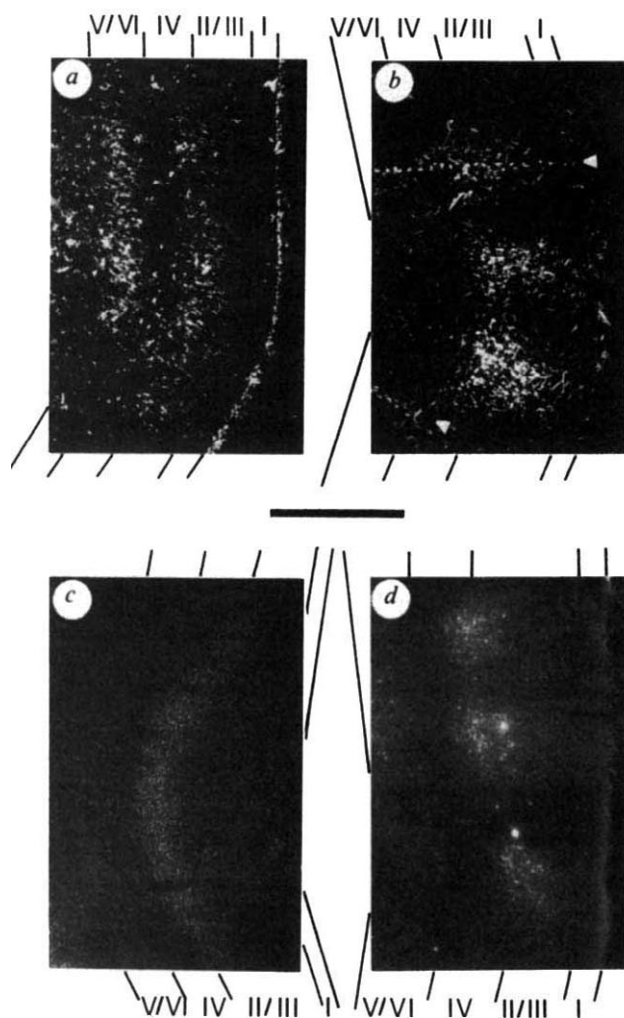


Fig. 2 Photomicrographs of area 17, showing labelling of neurones with FB or WGA-HRP after injections into area 18. *a*, At 8 h after injection of a 4-day-old kitten with WGA-HRP, showing labelled cells lying in continuous bands in the superficial and deep layers; *b*, 24 h after injection of a 20-day-old kitten with WGA-HRP in which labelled cells lie in patches mainly in the upper layers; *c*, 4 weeks after injection of a 2-day-old kitten with FB, showing persistent continuous bands of labelled cells that are much denser in superficial than in deep layers; *d*, 4 weeks after injection of a 2-month-old kitten with FB, showing clusters of labelled cells mainly in the superficial layers, with few in the deep layers. *a*, *b*, Dark-field-illuminated; *c*, *d*, fluorescence photomicrographs. Artefactual label is seen along the pial surface in *a* and *b*, and white arrowheads in *b* indicate radial blood vessels. Scale bar, 1 mm.

LGN, were probably less than 2 mm across. The positions of injection sites relative to the borders of area 18 were determined not only by direct examination of the cytoarchitectonics¹² and cytochrome oxidase activity¹³ of the surrounding cortex, but also by inspection of the distribution and size of labelled cells in the LGN. In all animals the group of labelled cells lay in the centre of the LGN (Fig. 1*a*) in the coronal plane, not extending to either the medial or lateral edges of the nucleus, and the average cross-sectional area of these neurones was considerably larger than the mean size of cells sampled from the total population in the same region of the nucleus (Fig. 1*b*), indicating that, in every case, a central portion of the width of area 18 had been successfully injected without involvement of area 17 or 19 (ref. 14).

Tracers were transported to the cell bodies of association neurones in a topographically corresponding part of area 17, in

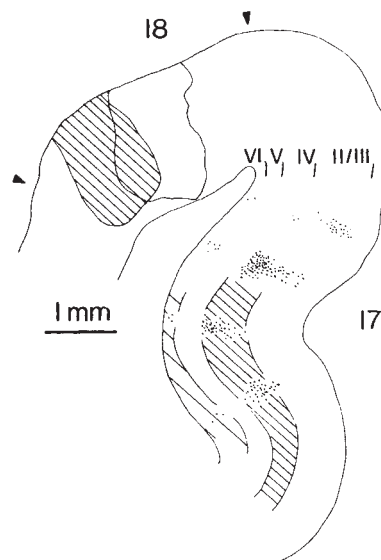


Fig. 3 Camera lucida drawing of a single coronal section through areas 17 and 18 of a 30-day-old kitten that had been injected with FB on day 2 and with DY on day 29. The FB injection site (cross-hatched) overlaps with the DY injection site (unshaded). FB-labelled cells in area 17 are found in two continuous bands (cross-hatched) in the superficial layers and, with lower density, in the deep layers, but DY-labelled cells lie in distinct clusters (dotted), mainly in superficial layers. Where these patches overlap with the band of FB-labelled cells, a number of the DY-labelled cells also contained FB. Areal boundaries were defined on the basis of Nissl- and cytochrome oxidase-stained material; cortical lamination is also indicated.

the same coronal plane as the injection site (Fig. 3); the position of this region of labelling provided yet more evidence that the injections had been placed in the body of area 18. The fact that a region of cortex between the injection and uptake sites contained no labelled cells (Fig. 3) indicated that tracer had not spread directly through the cortex to label neurones in area 17. Invasion of the white matter by diffused tracer around each injection site was slight and, in any case, the pattern of uptake in area 17 was different from that observed when white matter is deliberately injected².

In the kitten that was injected with FB at 2 months old and allowed to survive for 4 weeks, and in the 3-week kitten injected with WGA-HRP and perfused after 24 h (Fig. 2*d*, *b*), tracer was transported into dense clusters of cells in area 17 of ~300 µm diameter, separated by virtually label-free zones ~300 µm wide (Table 1 shows average cell densities in the middle of clusters in sections 50-µm thick). Just as in adult cats, most of the labelled neurones were in the upper cortical layers, with small numbers (one-fifth or less of the density of cells in the upper layers) lying in radially aligned sparse clusters in the lower layers (Table 1). The similarity in the patterns of labelling in area 17 in these two animals suggests that FB was not substantially metabolized and hence lost from cells during the weeks after an injection, nor did it spread widely to label distant cells. Previous workers have demonstrated that FB can remain for long periods within the cytoplasm of a neurone without obvious signs of damage or leakage across the cell membrane: even after several weeks, uptake by neighbouring cells (usually glial cells) is slight^{5,15}. We also saw occasional, very faintly stained cells near neurones that were heavily labelled with FB, but they were small in number, easily distinguished and were not included in our counts of labelled cells.

In younger kittens, aged 1, 4 and 10 days, that had been injected with WGA-HRP and perfused soon afterwards (8 h), the picture was quite different (Fig. 2*a*): there were two continuous bands of labelled cells of uniform density in area 17 in the 1- and 4-day animals, and in most sections from the 10-day

Table 1 Average densities of labelled cells (mm^{-2} , in sections $50\text{ }\mu\text{m}$ thick) in superficial and deep layers of area 17 after injections of tracers into area 18

	Injections followed by short (8 or 24 h) survival					Injections followed by long survival		
	Age at injection (days)					Age at injection		
	1	4	10	20	29	2 days	2 days	2 months
Cell density in superficial layers	170 (21)	462 (43)	239 (14)	363* (27)	390† (23)	331 (18)	375 (25)	382* (23)
Cell density in deep layers	209 (25)	210 (28)	191 (14)	52* (16)	55† (12)	81 (12)	69 (19)	74* (11)
Ratios of densities (superficial/deep)	0.8	2.2	1.3	7.0	7.1	4.1	5.4	5.2

Values are means (with s.e.m. in parentheses). In each of the animals in which labelled cells were distributed in non-patchy bands (that is, kittens injected at 1, 2, 4 and 10 days), 10 consecutive sections ($50\text{ }\mu\text{m}$ thick) through the centre of the area of uptake were selected and the cell density calculated in the upper and lower layers between two lines, 1–1.5 mm apart, drawn parallel to the radial palisades of cortical cells at the point where the density of labelled cells began obviously to decline towards the edge of the area of uptake. In those kittens in which a patchy distribution was seen, densities were measured within a number (* $n=15$; † $n=10$) of corresponding clusters in both superficial and deep layers from several sections covering the centre of the region of uptake. The ratios of the density in superficial layers to the density in deep layers are shown, and lie between 0.8 and 2.2 for all animals aged ≤ 10 days but are much higher in all kittens older than this at the time of perfusion, regardless of the age of injection.

animal. One band lay in superficial layers and the other band, of similar density, in deep layers (densities are shown in Table 1). In the youngest animals, the density of labelled cells was quite constant along each band and labelled cells were not scattered outside the area topographically related in position to the injection site. These facts argue strongly against the possibility that uptake of tracer by axons of passage in the cortex or the white matter had led to the aberrant pattern of labelling in area 17 in young kittens. In some sections from the 10-day-old animal there was evidence of patchiness in the distribution of labelled cells within the superficial and deep layers as the characteristic clustering of the association cells began to appear^{1,2}.

In the two 30-day-old animals that had been injected with FB on postnatal day 2, there was a non-patchy distribution of labelled cells in both superficial and deep layers in area 17 (Fig. 2c), although the density of labelled cells was markedly reduced in the deep layers (Table 1).

Thus, when a long-lasting tracer is injected into area 18 soon after birth, the pattern of label persisting in both superficial and deep layers of area 17 at a much later age remains non-patchy, as in the newborn kitten, despite the fact that the association projection has by this stage actually matured to its typical adult-like patchy form. When DY was injected into area 18 24 h before perfusion in a 29-day-old animal that had previously been injected with FB on day 2, a patchy pattern of DY-labelled cells was seen in addition to the continuous band of FB-labelled neurones in area 17 (Fig. 3). The centre of the DY injection was slightly closer to the 17/18 border than that of the FB injection, and the two stains only partly overlapped each other in area 18. The patches of DY-labelled cells in area 17 were correspondingly displaced towards the 17/18 boundary, compared with the band of FB-labelled neurones. Where the DY patches were superimposed on the continuous FB bands, about one-third of the labelled cells were clearly double-labelled.

As distinct, segregated patches of labelled cells in area 17 were still clearly visible 4 weeks after an injection of FB into a 2-month-old kitten, and since the density of label in 30-day-old animals injected with FB on day 2 was not uniform throughout the depth of the cortex but was virtually absent from the lower half of layer IV (Fig. 2), it is most unlikely that leakage and widespread diffusion of FB had obscured the appearance of patches in the latter animals.

We conclude that many of the cells in the superficial layers of area 17 (as well as some of those in the deep layers) of the neonatal kitten that initially send association projections survive, even though they lose their connections with area 18 by the third week postnatal, to leave the typical patchy distribution of cortico-cortical cells. Our results (Table 1) provide no evidence

for a net postnatal change in the density of association cells within regions of the upper layers of area 17 that remain connected to area 18, and indicate that wholesale death of association cells probably does not occur within the superficial layers. On the other hand, the density of labelled cells in the deep layers of area 17 was much reduced in older kittens and was very similar whether the injection into area 18 had been made on day 2, with long survival, or after 20 days of age. Thus, the loss of the aberrant early projection from the infra-granular layers may partly result from cell death. The marked reduction in cell density in deep layers relative to superficial layers (Table 1) cannot be explained by differential growth of the cortex. First, these observations were all made on the relatively flat portion of area 17, in the medial bank of the lateral gyrus, where areal expansion should stretch all layers roughly equally: in any case, direct measurement of the dimensions of the medial bank of the lateral gyrus suggests that this part of area 17 expands in surface area by only $\sim 15\%$ between days 10 and 20, during which period there is a fourfold or greater fall in the density of labelled cells in the lower layers (Table 1). Measurement of cortical layers II, III and upper IV shows that they actually increase in thickness (by 38% on average) more than do layers V and VI (by 25%) over the first 4 weeks postnatal. Wholesale migration of association neurones from deep to superficial layers is not excluded but is unlikely as very few labelled cells were ever seen in lower layer IV. On balance, our results are most parsimoniously explained by the death of aberrant association neurones in the deep layers.

The cortico-cortical projection from area 17 to area 18 is present and topographically arranged at birth, before the onset of visual stimulation, and is highly exuberant, much of it originating from aberrant populations of cortical neurones. Axonal retraction and cell death both appear to have major roles in the postnatal refinement of this projection, the former leading to the typical clusters of association cells in the upper layers, the latter contributing to the substantial reduction of the projection from the lower layers.

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Ultrastructural localization of choline acetyltransferase in vascular endothelial cells in rat brain

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Furchgott and Zawadski¹ have shown that acetylcholine (ACh) does not act directly on the smooth muscle of blood vessel walls, but rather via receptors on the endothelial cells lining the lumen, to release an endothelium-derived relaxing factor (EDRF). As it is very unlikely that neurotransmitter released from the periaxonal nerves, which are confined to the adventitial-medial border, diffuses all the way through the medial muscle coat before acting on endothelial cells to release EDRF to produce vasodilatation, this discovery has been regarded as an indication of a pathophysiological mechanism, rather than a physiological one (see refs 2, 3). ACh is rapidly degraded in the blood by acetylcholinesterase, so that ACh must be released locally to be effective on endothelial cells. Here we demonstrate the immunocytochemical localization of choline acetyltransferase in endothelial cells of small brain vessels, which is consistent with the view that the ACh originates from endothelial cells that can synthesize and store it. We suggest that release of ACh following damage to endothelial cells during ischaemia contributes to a pathophysiological mechanism of vasodilatation which protects that segment of vessel from further damage as well as brain cells from hypoxia.

The source of local ACh that might be involved in pathophysiological vasodilatation via endothelial cells is problematical, and it is this aspect that has been examined in the present

investigation of vessels of the brain, where potent ACh-induced vasodilatation has been shown to be dependent on the presence of intact endothelial cells⁴.

Eight adult Wistar rats were anaesthetized with ether and perfused via a cannula tied into the ascending aorta with 200 ml of fixative containing 4% paraformaldehyde, 0.1% glutaraldehyde, 0.1 M lysine-HCl and 0.01 M sodium periodate in 0.1 M phosphate buffer, pH 7.3. This was immediately followed by 500 ml of the same fixative without glutaraldehyde. The brains were removed and placed in fixative for 2 h, then transferred to 0.1 M phosphate buffer and stored overnight at 4 °C. Coronal sections through the visual cortex were cut at 50 µm using a Vibratome and collected in 0.1 M Tris buffer. Sections were processed for immunocytochemical staining of choline acetyltransferase (ChAT) using a recently derived rat monoclonal antibody against ChAT prepared and tested for specificity as described previously^{5,6}. Following treatment with 3,3'-diaminobenzidine and hydrogen peroxide, sections were transferred to 1% OsO₄ for 30 min, stained with 1% aqueous uranyl acetate, dehydrated through an ethanol series and embedded in Araldite. Preincubation of the antiserum with purified ChAT has been shown to abolish cortical staining⁷. In sections from two control rats the primary antibody or the rabbit anti-rat IgG were omitted, resulting in elimination of positive staining in the peroxidase-antiperoxidase procedure.

Examination of the sections under the light microscope and of numerous ultrathin sections from all experimental animals with the electron microscope showed that a small percentage (~10%) of endothelial cells of cortical capillaries and small blood vessels contained ChAT immunoreactivity (Fig. 1). This immunoreactivity was confined to the cytoplasm. Because of the dark nature of the reaction product, organelles present in the cytoplasm could not be discerned, except for mitochondria which appeared to be clustered near the nucleus (Fig. 1c). The relatively small number of labelled endothelial cells may indicate that the levels of ChAT are too low to be detected in some cells and/or that a small proportion of cells containing ACh is sufficient to activate neighbouring cells to release EDRF. ChAT immunoreactivity was also localized in nonpyramidal neurones primarily of the bipolar variety, in agreement with previous findings^{8,9}. Occasionally the dendrites of these neurones, as well as ChAT-stained axons, appeared to be in close association with blood vessels.

The finding that ChAT is localized in vascular endothelial cells in the brain prompts the speculation that choline is taken

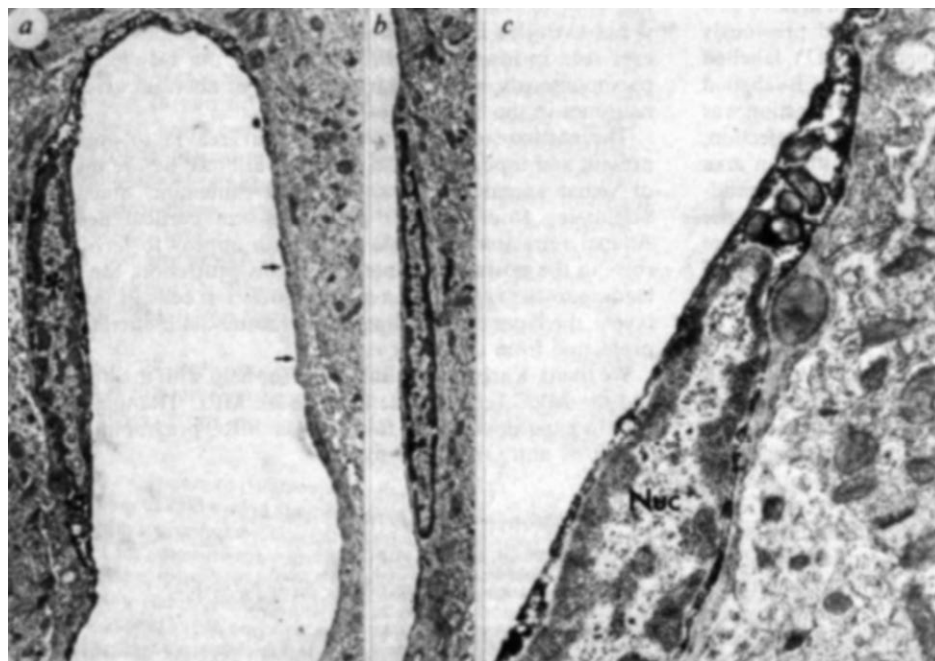


Fig. 1 *a*, Longitudinal section through part of a capillary in the upper layers of the rat visual cortex. The wall of the capillary comprises a ChAT-labelled endothelial cell (*) and a process (arrows) of an unlabelled endothelial cell from the same capillary. *b*, Unlabelled endothelial cell from the same capillary. *c*, The labelled cell shows a clear nucleus (Nuc) and thin perinuclear cytoplasm containing a few organelles and a cluster of mitochondria. *a*, *b*, $\times 5,830$; *c*, $\times 15,770$.

up by endothelial cells and converted in the presence of ChAT to be stored as ACh; any local damage to these cells would result in release of ACh and vasodilatation resulting from relaxation of the smooth muscle and/or endothelial cell processes (see ref. 10). Such local vasodilatation mediated by ACh released from damaged endothelial cells would provide protection from further damage in this region of the vascular bed and would protect brain cells from hypoxia.

The number of endothelial cells that contain ChAT in different vascular beds and vessels, and within particular vessels in any one bed, is currently being studied. Preliminary studies have revealed ChAT immunoreactivity in endothelial cells of mesenteric arteries of the rat, but it will be necessary to study ChAT localization in some larger vessels, such as the rabbit thoracic aorta, the tissue that Furchgott used when making the original observations, before it can be claimed that local synthesis, storage and release of ACh from endothelial cells is the universal

mechanism for ACh endothelial cell-mediated vasodilatation. It will also be important to determine how far the ACh released from endothelial cells is conveyed to other parts of the vasculature before being inactivated.

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Glial cells express N-CAM/D2-CAM-like polypeptides *in vitro*

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The joining together of neurites to form fascicles and the growth of axons along glial surfaces^{1,2} during early development suggest that neurone-neurone and neurone-glial adhesion interactions are of considerable importance for defining nerve tracts. *In vitro* studies have indicated that adhesion between neurones³⁻⁵ involves a glycoprotein that has been independently studied under the names of N-CAM (for neural cell adhesion molecule⁶⁻⁸), D2-CAM⁹ and BSP-2 (refs 10, 11). As N-CAM/D2-CAM appears to be a homophilic ligand¹² that binds to N-CAM/D2-CAM polypeptide on adjacent cells, this glycoprotein is potentially important in adhesion interactions between any two N-CAM/D2-CAM-expressing cells. While it has been suggested that neurone-glial adhesion involves molecules other than N-CAM/D2-CAM¹³⁻¹⁵, it is known that N-CAM/D2-CAM antigenic determinants are expressed by glial cells *in vivo*^{16,17} and that injection of anti-N-CAM antibodies into the eye-cup of chick embryos disrupts normal patterns of neuritic apposition to glial endfeet in the developing optic stalk¹⁷. Do the molecules expressed by glia share restricted antigenic determinants, or binding domains, with N-CAM/D2-CAM, or are N-CAM/D2-CAM polypeptides expressed by glia? Here we present immunocytochemical evidence which suggests that all classes of macroglia express N-CAM/D2-CAM antigenic determinants on their surfaces and immunochemical analyses which indicate that the molecules expressed by purified astrocytes are closely similar, or identical, to at least some forms of N-CAM/D2-CAM obtained from whole brain or purified neurones. However, our results also suggest that different N-CAM/D2-CAM polypeptides may be separately expressed by neurones and astrocytes.

Expression of N-CAM/D2-CAM antigenic determinants was examined in cultures of all classes of central nervous system (CNS) macroglia that can be identified with cell-type specific antibodies and on Schwann cells, the glial cells of the peripheral

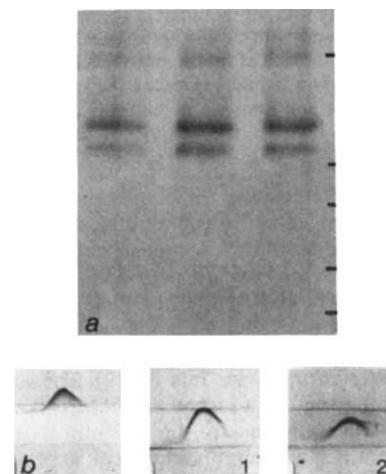


Fig. 1 *a*, Comparison of the anti-D2-CAM (lane 2), anti-N-CAM (lane 3) and anti-BSP-2 (lane 1) antisera in terms of the antigens immunoprecipitated by each from rat brain membranes indicates that all three antisera extract the same three polypeptides from brain. Bars represent the positions of standard markers of M_r : 200,000, 100,000, 69,000, 46,000 and 31,000. *b*, Antibody specificities were compared by crossed immunoelectrophoresis with intermediate gel. The intermediate gel contained no antiserum (left), anti-BSP-2 antiserum (middle) and anti-N-CAM antiserum (right).

Methods. *a*, Anti-D2-CAM antiserum is described in ref. 30 and anti-BSP-2 in ref. 27; anti-N-CAM antiserum was produced by injecting a rabbit with three intraperitoneal injections of 50 μ g of purified mouse N-CAM (purified by affinity chromatography using a monoclonal antibody specific for N-CAM carbohydrate structures²⁵ in Freund's complete adjuvant at 3-week intervals (M.W. and U.R., unpublished). Membranes (from 25-day-old animals) were labelled with ¹²⁵I by the chloramine-T method⁴⁵. Iodinated membranes were solubilized in Tris-barbitol buffer (0.07 M Tris, pH 8.6) containing 4% Triton X-100, 0.2 mM phenylmethylsulphonyl fluoride and 100 U ml⁻¹ aprotinin for 60 min on ice. After centrifugation at 10,000g for 15 min, the supernatants were submitted to crossed electrophoresis⁴⁶ with anti-BSP-2 (lane 1), anti-D2-CAM (lane 2) or anti-N-CAM (lane 3) antibody in the second-dimension gel. Immunoprecipitates were solubilized by boiling for 5 min in buffer containing 2% w/v SDS and 5% v/v 2-mercaptoethanol and analysed by SDS-polyacrylamide gel electrophoresis on 4-20% gradient gels⁴⁷. Gels were dried and fixed for autoradiography on Kodak X-Omat film at -70 °C. *b*, For crossed immunoelectrophoresis⁴⁸, Triton X-100 solubilized rat brain membranes were submitted to crossed immunoelectrophoresis against anti-D2-CAM antiserum. An intermediate gel was included containing the following antisera: (left) no antiserum, (middle) anti-BSP-2 antiserum, and (right) anti-N-CAM antiserum. After electrophoresis the plates were stained for protein with Coomassie stain. Anti-BSP-2 and anti-N-CAM antibodies retracted the D2-CAM immunoprecipitates into the intermediate gel, demonstrating that they recognize the D2-CAM antigen(s).

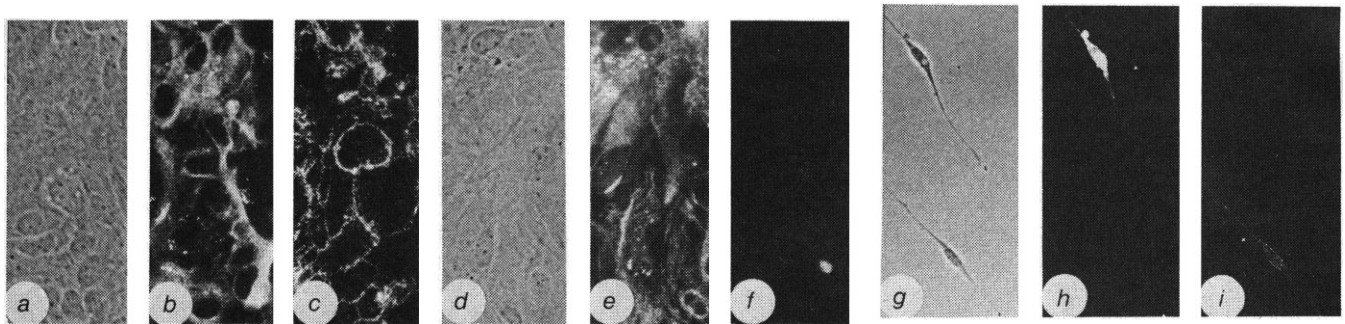


Fig. 2 N-CAM/D2-CAM antigenic determinants are expressed by astrocytes and Schwann cells. *a-c*, Living cultures of purified type 1 astrocytes^{18,19} grown on poly-L-lysine-coated glass coverslips were incubated first with H28 monoclonal antibody (hybridoma supernatant, undiluted), and then with biotin-conjugated goat anti-rat immunoglobulin (Amersham, 1:100), and fluorescein-conjugated streptavidin (Amersham, 1:100). Cultures were then fixed with methanol (-20°C , 10 min) and stained with rabbit anti-glial fibrillary acidic protein (GFAP) antiserum⁴⁹ (1:2,000), followed by rhodamine-conjugated goat anti-rabbit immunoglobulin (RIG-Rd). Identical staining patterns for N-CAM/D2-CAM were obtained by staining the mouse cultures, or similar rat astrocyte cultures, with rabbit anti-N-CAM, anti-D2-CAM or anti-BSP-2 antisera, followed by labelling with goat anti-RIG-Rd. *a*, Phase contrast; *b*, anti-GFAP; *c*, H28 anti-N-CAM. *d-f*, Control staining for *a-c*. H28 antibody was not included in the antibody incubations. *g-i*, Schwann cells from the sciatic nerves of neonatal mice were examined with H28 antibody and with rabbit anti-N-CAM, D2-CAM or BSP-2 antisera. Cultures were labelled as for *a-f*, except that rabbit anti-P₀ antiserum was used in place of rabbit anti-GFAP. N-CAM/D2-CAM antigenic determinants were expressed by many Schwann cells; however, most Schwann cells which had expressed the myelin protein P₀ did not co-express N-CAM/D2-CAM. *g*, Phase contrast; *h*, anti-P₀; *i*, H28. Labelling conditions and analysis of cultures as in refs 18-20. Serum from nonimmune rabbits did not label the surface of astrocytes or Schwann cells, and none of the conjugates used reacted with antibodies from inappropriate species or labelled cells in the absence of anti-N-CAM/D2-CAM or cell-type-specific antibodies.

nervous system. Purified astrocytes from rat and mouse cerebral cortex were prepared according to published procedures^{18,19}. These cultures contained 95-98% type 1 astrocytes²⁴—that is, 95-98% of the cells were labelled with antiserum against glial fibrillary acidic protein (GFAP), a cell-type specific marker of astrocytes²¹, but did not label with the monoclonal antibody A2B5 (ref. 22). These cultures contained no other classes of macroglia, and no neurones (see refs 18, 19). The small proportion of contaminating cells were fibroblast-like cells that expressed cell-surface fibronectin, but did not express GFAP. All immunolabelling and immunochemical experiments were carried out on astrocyte cultures of this kind. In addition, experiments involving biosynthetic labelling or immunochemical quantification were repeated on astrocyte cultures that were prepared by other methods²³ and grown for 3-4 weeks in Dulbecco's modified Eagle's medium containing 10% v/v heat-inactivated horse serum. The two types of astrocyte cultures produced identical results in these experiments. Expression of N-CAM/D2-CAM determinants by other CNS glia was examined in cultures of optic nerves from embryonic chicks and neonatal rats and mice, and expression of these determinants by Schwann cells was examined in enriched Schwann cell cultures prepared from sciatic nerves of neonatal mice (as in ref. 24).

Cultures were examined with three separate rabbit antisera (anti-N-CAM, anti-D2-CAM and anti-BSP-2, see Fig. 1) against the N-CAM/D2-CAM polypeptides, with a monoclonal antibody directed against chick N-CAM²⁵ and the H28 monoclonal antibody directed against mouse N-CAM^{11,26}. N-CAM/D2-CAM is expressed in multiple forms that differ in their carbohydrate composition^{6-8,10} and the size of their cytoplasmic domains²⁷, but that are thought to be otherwise identical^{27,28}. In post-embryonic brain the three dominant forms of this molecule have relative molecular masses (M_r) of 120,000, 140,000 and 180,000 (or 200,000, depending on the gel system used)^{26,29,30}. The three rabbit antisera used in our studies immunoprecipitate all three of these polypeptides from rat brain extract (Fig. 1*a*), both anti-N-CAM and anti-BSP-2 antisera react with the D2-CAM antigens (Fig. 1*b*), and all reactivity of N-CAM/D2-CAM antigen(s) with anti-D2-CAM antisera could be removed from extracts of rat brain by preabsorption with anti-N-CAM or anti-BSP-2 antisera (not shown). Thus, it appears that these three antisera are all directed against identical N-CAM/D2-CAM polypeptides. Both monoclonal antibodies have been previously characterized as recognizing protein determinants specific for the N-CAM/D2-CAM polypeptides^{25,31}.

Using immunofluorescence microscopy, all classes of glia examined were found to express N-CAM/D2-CAM determinants. In cultures of purified type 1 astrocytes from rat cerebral cortex, all of the GFAP⁺ astrocytes were labelled by all three rabbit antisera; purified type 1 astrocytes from mice were similarly labelled, and were also labelled by the H28 monoclonal anti-N-CAM antibody (Fig. 2). In cultures of optic nerve from neonatal rats, examined 1 and 3 days after preparation of the cultures, the rabbit antisera labelled cells possessing the antigenic and morphological phenotypes of oligodendrocytes, type 2 astrocytes, oligodendrocyte-type 2 astrocyte (O-2A) progenitors^{20,32} and type 1 astrocytes (not shown). Cells in cultures of mouse optic nerve were similarly labelled and were labelled by the H28 monoclonal anti-N-CAM antibody (not shown). In cultures of chick optic nerve, the majority of cells were labelled with monoclonal anti-chick-N-CAM antibody (not shown). As optic nerve cultures contain no neurones³², these results suggest that N-CAM/D2-CAM determinants are expressed by all of the major classes of CNS macroglia which can be identified by cell-type specific antibodies, and that these determinants are either expressed *in vivo* or are expressed within 24 h of growth *in vitro*. In addition, in cultures of enriched Schwann cells from sciatic nerves of 3-7-day-old mice (prepared as in ref. 24), we found labelling of some, but not all, cells with the morphology typical of Schwann cells (Fig. 2). In double-labelling experiments using rabbit antiserum against the myelin protein P₀, which labels Schwann cells that have been induced to synthesize myelin-specific proteins³³, many of the P₀⁺ Schwann cells were not labelled by any of the rabbit anti-N-CAM/D2-CAM antisera, or by the H28 monoclonal antibody. Some double-positive cells were present in the cultures, but the majority of N-CAM/D2-CAM⁺ cells with Schwann-cell like morphologies were P₀⁻. Loss of expression of N-CAM/D2-CAM antigenic determinants by myelinating Schwann cells has been seen *in vivo*³⁴, and a comparable loss of N-CAM/D2-CAM expression by older oligodendrocytes has also occasionally been seen *in vitro* (M.N. and I. Sommer, independent unpublished observations). In cultures of sciatic nerve that had not been treated to enrich for Schwann cells, a small proportion (<20%) of cells with fibroblastic morphology were lightly labelled by the H28 monoclonal anti-N-CAM/D2-CAM antibody, but most fibroblast-like cells in these cultures were N-CAM/D2-CAM⁻.

Immunochemical analyses further support the view that the determinants recognized by indirect immunofluorescence, at least on Type 1 astrocytes, represent N-CAM/D2-CAM. (1) Immunoblots of astrocyte extracts with both anti-N-CAM and

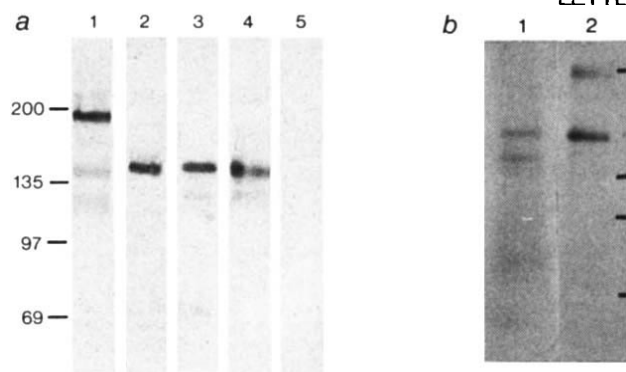


Fig. 3 *a*, SDS-gel electrophoresis of 7-day neonatal mouse brain (1), rat retina (2) or cultured astrocytes (3-5) followed by immunoblotting with anti-N-CAM (1-3), anti-D2-CAM (4) or nonimmune (5) antisera. *b*, Immunoprecipitation of N-CAM/D2-CAM polypeptides from astrocytes and neurones radiolabelled *in vitro* with ^{35}S -methionine. Lane 1, astrocyte cultures; lane 2, cerebellar neurone cultures. *a*, Purified astrocytes were scraped off the surface of Falcon flasks (no. 3024) on ice and centrifuged at 500g for 10 min at 4°C. The cell pellet, or brain tissue, was solubilized in an equal volume of extraction buffer containing 2% SDS and 5% 2-mercaptoethanol, subjected to electrophoresis in 7.5% acrylamide gels, and transferred to nitrocellulose sheets⁵⁰. N-CAM/D2-CAM was visualized by reaction with the appropriate antiserum followed by horseradish peroxidase-conjugated goat anti-rabbit IgG and staining by treatment with 4-chloro-1-naphthol⁵¹. M_r s in SDS are based on the migration of myosin, phosphorylase *b*, bovine serum albumin and ovalbumin. Staining is seen of material of M_r 140,000, with very light staining of material of M_r 120,000. *b*, Cultures were labelled with 500 μCi of ^{35}S -methionine (NEN) for a 30-min labelling period, followed by a 30-min chase. Cells were collected, solubilized with Triton X-100 and N-CAM/D2-CAM antigens were immuno-isolated by immunoelectrophoresis against anti-D2-CAM antiserum³⁸. Cultures of cerebellar neurones were established from 8-day-old rats as described by Meier and Schousboe⁵², except that the medium contained 10% v/v horse serum instead of fetal calf serum. After 2 weeks the cultures were labelled with ^{35}S -methionine as described for astrocyte cultures, except that both labelling and chase periods were of 60 min duration. Cells were collected and D2-CAM immuno-isolated as above. Immunoprecipitates were further analysed by SDS-polyacrylamide gel electrophoresis as described in Fig. 1a.

anti-D2-CAM antisera recognized a predominant band of M_r 140,000 that migrated in the same position as the 140,000- M_r band from brain. A weak staining was also observed at M_r 120,000, but this did not reproduce well in the photography (Fig. 3a). (2) In a second protocol, astrocytes and purified neurones³⁵ were separately labelled *in vitro* with ^{35}S -methionine, anti-D2-CAM antiserum was used to immunoprecipitate radiolabelled antigens³⁵, and precipitates were analysed on SDS-polyacrylamide gels (Fig. 3b). This procedure yielded two predominant astrocytic polypeptides, one band of M_r ~140,000, which migrated identically with one of the N-CAM/D2-CAM bands isolated from neuronal cultures, and a second band of M_r ~120,000. A faint band of higher relative molecular mass, which in the gel system used in these particular experiments was of M_r ~200,000, was occasionally observed in extracts prepared from purified astrocytes. N-CAM/D2-CAM immuno-isolated from purified neurones in culture (Fig. 3b, see also ref. 35) also consisted of two bands, but these were of M_r 140,000 and 200,000; no band of M_r 120,000 was detected in neuronal extracts. The polypeptide of M_r 200,000 seen in neuronal extracts is the same polypeptide which in other gel systems, such as that used in Fig. 3a, has a M_r of ~180,000. Thus, in most experiments we were only able to identify the three N-CAM/D2-CAM polypeptides typical of whole brain (M_r = 120,000, 140,000 and 180,000-200,000^{26,29,30}) by considering the bands obtained from astrocytes and neurones in combination. The apparent lack of neuronal synthesis of the 200,000- M_r band

of N-CAM/D2-CAM has also been seen in recent *in vivo* studies in which chick retinal ganglion neurones have been found to transport N-CAM/D2-CAM polypeptides of M_r 140,000 and 180,000, but not of M_r 120,000, along the optic nerve (J. A. Gardner, M.W. and U.R., manuscript in preparation). There is evidence to suggest that different bands of N-CAM/D2-CAM might be transcribed from different messenger RNAs³⁶; it will now be of interest to compare the details of N-CAM/D2-CAM structure, synthesis and processing in neurones and astrocytes.

From the present studies we conclude that glial cells express N-CAM/D2-CAM antigenic determinants on their surfaces, and that purified type 1 astrocytes grown *in vitro* synthesize N-CAM/D2-CAM-like polypeptides. The astroglial molecules we have examined appear to be closely related to N-CAM/D2-CAM polypeptides characterized in extracts of whole brain, and are probably not related to two other putative cell adhesion molecules from the central nervous system, Ng-CAM^{13,14} and L1 (ref. 37), which are thought to be expressed by neurones but not by CNS glia. The three N-CAM/D2-CAM polypeptides isolated from whole brain are thought to differ primarily in the length of their cytoplasmic domains²⁷, and have previously been found to be almost identical as judged by peptide mapping^{27,28,38}. In contrast, peptide maps of L1 and Ng-CAM are markedly different from those of N-CAM/D2-CAM^{14,39}. L1 and Ng-CAM are also immunochemically distinct from N-CAM, although there may be some shared expression of at least one epitope that has been identified by monoclonal antibodies^{14,40}. Moreover, although L1 has an M_r of ~140,000 and Ng-CAM an M_r of 135,000, which are similar in size to one of the astroglial N-CAM/D2-CAM bands, L1 and Ng-CAM antibodies do not detect material of M_r 120,000, the size of the second major astroglial N-CAM/D2-CAM band.

Although in our present studies we have consistently seen high levels of N-CAM/D2-CAM expression by a variety of glial cell types, previous investigations have suggested that glia do not express N-CAM/D2-CAM *in vitro*^{13,14,26}. We do not yet know the reasons for these differing results; however, it seems likely that expression of N-CAM/D2-CAM by astrocytes can be modulated during development, as appears to be the case for muscle cells^{41,42} and Schwann cells. Nothing is yet known about the regulatory influences that might induce the disappearance of N-CAM/D2-CAM from astrocytes.

The hypothesis that neurones are more adherent to glia than to other neurones or to non-glia has been suggested as providing a cellular explanation for the observations that, both *in vivo* and *in vitro*, growing neurites exhibit a marked affinity for glial surfaces^{18,43,44}. For example, during embryonic development of the optic stalk, neuritic growth cones are found predominantly apposed to endfeet of primitive glia and neuritic fascicles are found organized on these glial surfaces but not on other available surfaces¹. The demonstration of N-CAM/D2-CAM on glial cell surfaces raises the question of whether this molecule is involved in such adhesive interactions between neurones and glia, in distinction from the hypothesis that neurite-neurite adhesion is mediated by N-CAM/D2-CAM and neurone-glia adhesion is separately mediated by Ng-CAM^{14,15}. It has recently been found that N-CAM/D2-CAM antigenic determinants are expressed on glial endfeet in the chick optic stalk and that injection of anti-N-CAM antibody into the eye-cup of chick embryos results, within the optic stalk, in a separation of neuritic fascicles from these glial surfaces¹⁷. Thus, in this particular case, the affinity of neurites for glial surfaces may be due to glial expression of N-CAM/D2-CAM. However, the specific contributions of N-CAM/D2-CAM, Ng-CAM and perhaps other molecules to the generally observed affinity between neurones and glia have yet to be determined.

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Note added in proof: Independent experiments by G. Keilhauer, A. Faissner and M. Schachner (*Nature* 316, 728-730; 1985) have also indicated that purified astrocytes express N-CAM/D2-CAM-like in polypeptides. These experiments also indicate that the binding of neurones to astrocytes *in vitro* can be inhibited by anti-N-CAM/D2-CAM antiserum.

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Differential inhibition of neurone-neurone, neurone-astrocyte and astrocyte-astrocyte adhesion by L1, L2 and N-CAM antibodies

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The cell adhesion molecules L1, N-CAM and Ng-CAM have been implicated in cell-cell interactions among developing neural cells¹⁻⁴. L1 and N-CAM are structurally and functionally distinct molecular entities and act synergistically in mediating Ca²⁺-independent adhesion between re-aggregating early postnatal cerebellar cells⁵. N-CAM has been reported to be neurone-specific in the chicken and to mediate fasciculation of neurites and of nerve-muscle interactions. L1, which in the central nervous system has been found only on post-mitotic neurones, mediates migration of granule cell neurones in the mouse cerebellar cortex⁶. In view of the molecules' distinct effects on cell interactions, we wondered whether different neural cell types are involved in the actions of each molecule. Here we report that L1 antigen promotes neurone-neurone adhesion. N-CAM, which is expressed on both neurones and glia, mediates neurone-neurone, neurone-astrocyte and astrocyte-astrocyte adhesion. The L2 carbohydrate epitope shared between the two adhesion molecules⁷ seems to be involved in neurone-astrocyte and astrocyte-astrocyte adhesion and acts in a more than additive manner in N-CAM-mediated neurone-neurone adhesion.

Homogeneous populations of neurones and astrocytes from early postnatal mouse cerebellum were isolated, probed for expression of adhesion molecules (Figs 1, 2) and used to measure adhesion of single-cell suspensions of probe to monolayer target cells in the absence and presence of Fab fragments (Table 1). In all combinations of probe to target cells (neurone-neurone, neurone-astrocyte, astrocyte-neurone and astrocyte-astrocyte),

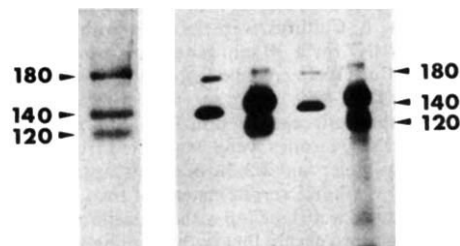


Fig. 1 Double immunofluorescence labelling of monolayer cultures from 5-day-old C57BL/6J mouse cerebellum after 2 days *in vitro*. Polyclonal N-CAM antibody (a), prepared as described previously³, reacts with vimentin-positive (b), fibronectin-negative glial cells. Note the patchy distribution of N-CAM on the cell surface (a). c, Phase-contrast micrograph of fluorescence images a and b. Polyclonal N-CAM antibody (d) reacts with an O4 antigen-positive oligodendrocyte (e). f, Phase-contrast micrograph of fluorescence images d and e. Cell culture, cell type-specific markers and double immunofluorescence procedures have been described previously¹⁹. Immunofluorescence labelling of GFAP-positive astrocytes was weaker than that of less differentiated, GFAP-negative, vimentin-positive astrocytes. The tendency for N-CAM to be more strongly expressed on less differentiated glia was also seen with oligodendrocytes: more than 80% of all O4 antigen-positive cells, but only less than 5% of all O1 antigen-positive cells were N-CAM-positive. $\times 300$.

antibodies to liver membranes, which react well with cell surfaces of astrocytes and neurones⁶, did not inhibit adhesion, whereas N-CAM antibodies did. Polyclonal L1 antibodies interfered with neurone-neurone adhesion, but not with neurone-astrocyte or astrocyte-astrocyte adhesion, even when astrocytes from different postnatal ages, from different brain regions or maintained *in vitro* for varying time periods were used. Monoclonal L2 antibodies did not reduce neurone-neurone adhesion, but decreased neurone-astrocyte and astrocyte-astrocyte adhesion. Astrocyte-neurone adhesion resulted in slightly different inhibition values from those seen in the reciprocal combination, as has been observed in other adhesion systems⁸.

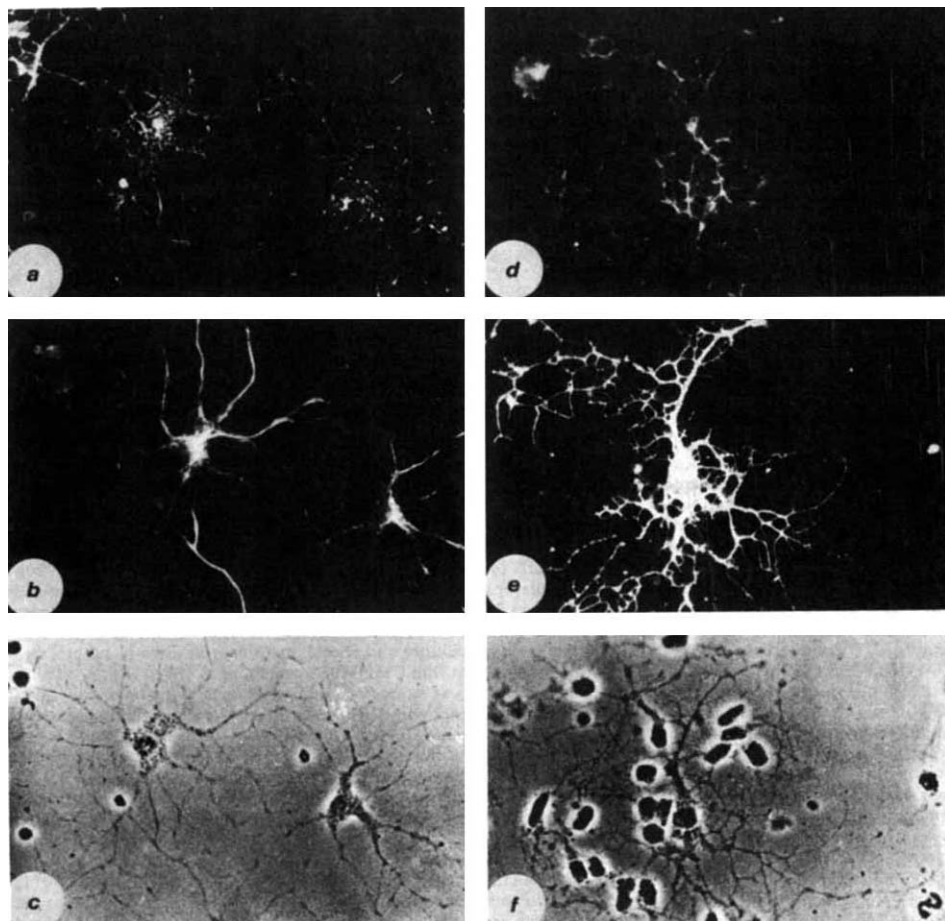
Inhibition values obtained with mixtures of polyclonal L1

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Fig. 2 Immunoprecipitation of N-CAM from cultures of isolated neurones and astrocytes from early postnatal mouse cerebellum using poly- and monoclonal N-CAM antibodies. *a*, Western blot with polyclonal N-CAM antibody ($10 \mu\text{g ml}^{-1}$ IgG fraction) on crude membrane fraction from adult mouse brain resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on a 6–12% gradient gel². Three bands are seen in the relative molecular mass regions of 180,000 (180K), 140K and 120K. *b–e*, Fluorograph of immunoprecipitates from ³⁵S-methionine-labelled cultures of neurones (*b, d*) and astrocytes (*c, e*) with polyclonal N-CAM (*b, c*) and monoclonal N-CAM antibody H28.123 (*d, e*). Polyclonal and monoclonal antibodies gave identical results. In neurone cultures the antibodies detected two bands of 180K and 140K. In astrocyte cultures two prominent bands in the 140K and 120K range were seen and a fainter band in the 180K range. Note the apparently higher molecular weight of the 180K and 140K forms in astrocytes compared with neurones.

Methods. Cells were obtained and maintained as described in the legend to Table 1. Cultures of 1.5×10^7 cells per 6-cm Petri dish were incubated with $200 \mu\text{Ci } ^{35}\text{S}$ -methionine (Amersham; $1,000 \text{ mCi mmol}^{-1}$) per ml in methionine-free minimal essential medium containing 10 mM HEPES and 2 mM glutamine for 4 h at 37°C .

After two washes with HBSS, cells were solubilized by adding 1 ml of solubilization buffer (150 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 10 mM methionine, 0.5% Nonidet P-40, 0.05% NaN_3 , pH 7.4), the protease inhibitors aprotinin, soybean trypsin inhibitor and turkey egg white trypsin inhibitor (all at $10 \mu\text{g ml}^{-1}$), phenylmethylsulphonyl fluoride and iodoacetamide (both at 5 mM), pepstatin and leupeptin (both at $2 \mu\text{M}$). After 30 min on ice, cells were scraped on to the dish, nuclei and debris removed by centrifugation for 10 min at 800g and supernatants cleared for 45 min at 100,000g. For immunoprecipitation, aliquots (1 ml) of the supernatants were incubated with polyclonal N-CAM (150 μg IgG fraction) or monoclonal N-CAM antibody H28.123 (ref. 23; $10 \mu\text{g}$ IgG fraction) for at least 1 h at 4°C . Then 150 μl protein A-Sepharose (10% v/v; Sigma) was added to rabbit IgG and 70 μl of a 10% v/v suspension of Sepharose 4B beads coupled with mouse monoclonal antibody to rat κ -chain²⁴ to monoclonal H28.123 antibody, all in solubilization buffer. After 1 h at 4°C , the beads were centrifuged through a sucrose cushion, washed several times with solubilization buffer and boiled for 4 min at 100°C in 45 μl of SDS-PAGE sample buffer⁵. All steps were carried out at 4°C . Precipitates were analysed by SDS-PAGE on 6–12% gradient gels⁵. Gels were then fixed in 10% acetate, 30% methanol in water and treated with 'amplify' as recommended by the producer (Amersham). Kodak X AR-5 X-ray films were exposed for various times at -70°C and developed according to the supplier's instructions.



and N-CAM antibodies were not additive, indicating that the two adhesion mechanisms are not independent in this adhesion system. In the presence of L2 antibody, polyclonal N-CAM antibodies were more than additive in inhibiting neurone-neurone, but not neurone-astrocyte or astrocyte-astrocyte adhesion. In contrast, inhibition by L2 antibody was unaffected by monoclonal N-CAM antibody, which by itself did not interfere with adhesion (not shown, see also ref. 3). Also, L2 antibody did not increase polyclonal L1 antibody-induced inhibition of neurone-neurone or neurone-astrocyte adhesion (not shown). In the presence of the three antibodies—polyclonal N-CAM and L1 antibodies and monoclonal L2 antibodies (each at a concentration of 1.0 mg ml^{-1})—the inhibition of neurone-neurone adhesion did not exceed the values obtained with N-CAM and L2 antibodies alone. Adhesion of neurones and astrocytes to skin fibroblasts was not reduced by N-CAM, L1 or L2 antibodies, regardless of whether fibroblasts were used as target or probe cells.

Our study has shown that distinct adhesion mechanisms operate between neurones and astrocytes in the mouse cerebellum at the time of postnatal granule cell migration: (1) L1 antigen is involved in neurone-neurone interaction. (2) N-CAM not

only mediates neurone-neurone, but also neurone-astrocyte and astrocyte-astrocyte adhesion. All cell types involved express N-CAM on their surfaces. (3) The L2 domain is involved in an adhesive function that is manifest by itself in neurone-astrocyte and astrocyte-astrocyte and, in conjunction with N-CAM, in neurone-neurone adhesion.

Neurone-glia interaction has been postulated to underlie the migration of granule cell neurones in the early postnatal cerebellum⁹. Because L1 antibodies interfere with migration⁶ and seem to block only neurone-neurone adhesion, it is possible that a prerequisite for migration is an L1-mediated sorting out of post-mitotic granule cell bodies or extension of granule cell axons. On the other hand, it is conceivable, although unlikely, that the cultured neurones and astrocytes used in the present study are not representative of their *in vivo* counterparts. Although L1 antigen shares certain properties with the neurone-neurone and neurone-glia adhesion molecule Ng-CAM^{4,10}, the present study failed to detect the molecular domains involved in neurone-glia adhesion. It is therefore conceivable that the polyclonal L1 and Ng-CAM antibodies recognize different sets of functional sites on L1 antigen from mouse and Ng-CAM from chicken.

Table 1 Inhibition of adhesion between enriched populations of neurones and astrocytes from early postnatal mouse cerebellum in the presence of Fab fragments from L1, N-CAM and L2 antibodies

Antibody	Ref.	Fab concentration (mg ml ⁻¹)	Neurone* to neurone†	Neurone* to astrocyte†	Astrocyte* to astrocyte†	Astrocyte* to neurone†
None		0	0±3	0±2	0±2	0±4
Poly-liver	5	1.0	2±4	-3±2	1±1	-2±3
Poly-L1	8	1.0	27±4	0±3	-1±2	0±2
Poly-N-CAM	3, 4	1.0	40±7	29±7	24±2	28±5
		2.0	41±3	32±3	23±4	26±12
Mono-L2	6	1.0	1±5	25±10	15±3	15±8
Poly-L1 + poly-N-CAM		0.5±0.5	36±4	ND	ND	ND
		1.0±1.0	36±2	ND	ND	ND
Poly-N-CAM + mono-L2		1.0±1.0	56±3	34±6	26±3	ND

Single-cell suspensions from early postnatal C57BL/6J mouse cerebella¹⁹ were used for isolation of astrocyte- and neurone-enriched cell populations. Small cerebellar neurones were obtained from 6–8-day-old mice by centrifugation (10 min, 4°C, 1,200g) through Percoll (Pharmacia, 40.5% in Ca²⁺- and Mg²⁺-free Hank's balanced salt solution (CMF-HBSS) containing 10 mM HEPES and 0.1% bovine serum albumin pH 7.2). The pellet of cells was washed once in basal Eagle's medium with Earle's salts containing 10% horse serum (culture medium) and seeded in poly-L-lysine-coated Costar plates at a density of 1×10^6 in 0.5 ml culture medium per well. Monolayers were used as target cells after 3 days in culture with >99% of all cells expressing N-CAM or L1 antigen and ~60% the L2 epitope. The percentage of glial fibrillary acidic protein (GFAP)- and vimentin-positive astrocytes after 3 days in culture was <1%, compared with 6±2% GFAP-positive and 8±2% GFAP-positive and 8±2% vimentin-positive astrocytes in cultures made from whole cerebellum. Astrocytes were obtained from 3–5-day-old mice by seeding single-cell suspensions into T75 tissue culture flasks (Nunc) at a density of 30×10^6 cells in 10 ml culture medium per flask. After 3 days, cultures were treated with cell-surface-reactive monoclonal antibodies M5 directed against all cerebellar neurones (C. Lagenaur and M.S., in preparation), 04 directed against more and less differentiated oligodendrocytes²⁰, and MESA-1 directed against fibroblast-like cells of endothelial origin²¹ in the presence of guinea pig complement (diluted 1:10). Residual cells were treated with 100 µg ml⁻¹ trypsin, 1 mM EDTA in CMF-HBSS for 5 min at room temperature, centrifuged (5 min, 4°C, 60g) and seeded in poly-L-lysine-coated Costar plates (10^5 cells in 0.5 ml culture medium per well). Monolayer cultures were used as target cells after 1 day in culture with >97% of all cells expressing GFAP and N-CAM and ~60% the L2 epitope. Fibroblasts were obtained from the skin of 1-day-old mice by treatment with collagenase IV and trypsin²². No significant cell death or proliferation was observed within the culture periods studied. Probe cells were obtained from monolayer cultures of enriched neurones (after 1 day in primary culture), astrocytes (1 day after immunocytolysis and subsequent subculture) and fibroblasts (3 days in primary culture) by treatment with 20 µg ml⁻¹ trypsin, 1 mM EDTA in CMF-HBSS for 15 min at room temperature after three washes with CMF-HBSS containing 1 mM EDTA. To vitally stain the probe cells, fluorescein diacetate (10^{-6} M) was included during the trypsinization step. Trypsinization was stopped by adding 40 µg ml⁻¹ soybean inhibitor and cells were collected by centrifugation (10 min, 40°C, 60g). Treatment with the low trypsin concentration did not visibly reduce the intensities of immunofluorescence staining with N-CAM, L1, L2 and liver membrane antibodies compared with non-trypsinized cells. Cell concentrations were adjusted to 4×10^5 cells ml⁻¹ CMF-HBSS for neurones, and to 1×10^5 cells ml⁻¹ for astrocytes. Probe and target cells were treated with 0.5 ml Fab fragments of antibodies in CMF-HBSS for 20 min on ice before the adhesion test. Target cells were washed three times with CMF-HBSS containing 1 mM EDTA before addition of Fab fragments. Probe cells (2×10^5 neurones or 0.5×10^5 astrocytes in 0.5 ml) were added to target cells in Costar wells and incubated for 30 min at room temperature in a reciprocal shaker (Infors, Basel, Switzerland) at 40 cycles per min. Unbound probe cells were removed from the monolayer target cells by three gentle washings in CMF-HBSS (1 ml for each well and washing). Aggregation among probe cells was negligible both in controls and in the presence of Fab fragments. Adhering cells were scored by counting ~1,000 cells in >10 microscopic fields at $\times 100$ magnification with an inverted fluorescence microscope (ICM 405, Zeiss). Without Fab fragments the binding of the total input cells was $79 \pm 3\%$ (897 ± 34.0 cells mm⁻²) for neurone-neurone, $82 \pm 2\%$ (931 ± 22.7 cells mm⁻²) for neurone-astrocyte, $47 \pm 4\%$ (133 ± 11.3 cells mm⁻²) for astrocyte-astrocyte, and $49 \pm 2\%$ (138 ± 5.6 cells mm⁻²) for astrocyte-neurone adhesion. Using fibroblasts, $10 \pm 5\%$ binding was seen in all combinations with neurones and astrocytes. Binding of probe cells was predominantly to target cells and not to the cell-free substrate. Each experimental value was run in quadruplicate. Per cent inhibition of adhesion in the presence of Fab fragments was calculated by: % inhibition = [adhesion (control) - adhesion (+Fab)]/adhesion (control) $\times 100$. Numbers are mean values for several experiments $\pm$ s.d. Eight experiments were performed for neurone-neurone and neurone-astrocyte, and four experiments for astrocyte-neurone and astrocyte-astrocyte adhesion. The difference in inhibition of adhesion given by antibody combinations and individual antibodies is significant for the combination polyclonal N-CAM and monoclonal L2 only for neurone-neurone adhesion ($P < 0.001$, Student's *t*-test). Specifications of antibodies and preparations of Fab fragments have been described previously and appropriate references are given. ND, not done.

* Probe cells; † target cells.

N-CAM has previously been detected on glia in addition to neurones (refs 3, 11, 12; see also accompanying paper¹³), although its function in neurone-glia adhesion has remained elusive¹⁰. As N-CAM has been implicated in a homophilic 'self-aggregating' adhesion mechanism¹⁴, it is expected that N-CAM-positive glial cells adhere to other N-CAM-positive cells. In the conditions of our study, we did not observe the synergistic effect between L1 and N-CAM molecules which was seen in re-aggregating cultures of single cells⁵. It is likely that the non-additive effects are the result of different cell dissociating procedures, the lower temperature used to prevent self-aggregation among probe cells, and the anchoring of the target cells at the substrate surface, thereby reducing the molecules' lateral mobility within the membrane and cooperative effects between them.

Adhesion affinities of N-CAM are differentially modulated by the carbohydrate chains of the adult and embryonic forms^{14,15}. It has been suggested that carbohydrate chains are involved in stabilizing the conformation of glycoproteins, and thus indirectly affect their function^{16,17}, or that they are more directly involved as ligands for lectin-like receptors¹⁸. The L2 domain may be yet another, functionally important carbohydrate moiety. The function of the L2 epitope on some but not all N-CAM molecules of the adult and embryonic forms remains to be elucidated in the intact developing nervous system.

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Functional reconstitution of purified muscarinic receptors and inhibitory guanine nucleotide regulatory protein

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Muscarinic receptors trigger several different responses including an increase in concentration of cyclic GMP, a decrease in cyclic AMP concentration, breakdown of polyphosphoinositides and changes in ion permeability. It is not yet clear whether these reactions occur sequentially or independently and which directly coupled to the muscarinic receptor. Several lines of evidence indicate that muscarinic receptors in many, if not all, cell types are coupled to the inhibitory guanine nucleotide regulatory protein (N_i or G_i) of adenylate cyclase. To provide direct evidence for this coupling, we have reconstituted muscarinic receptors purified from porcine brain with N_i purified from rat brain in a phospholipid vesicle. Here, we report that the GTPase activity of N_i is stimulated by carbachol. This action is blocked by the simultaneous addition of atropine and is not observed when the N_i protein is ADP-ribosylated. We conclude that one function of the muscarinic receptor is the activation of N_i .

Either the receptor-linked inhibition of membrane adenylate cyclase or the decrease in cellular cAMP is reversed¹⁻³ by islet-activating protein (IAP, pertussis toxin⁴⁻⁶), which abolishes the function of N_i ^{2,7-9} as a result of ADP-ribosylation of the protein^{10,11}. Guanine nucleotides also markedly reduce the apparent affinity of membrane receptors for agonists but not for antagonists¹²⁻¹⁵, and the effect of guanine nucleotides is abolished by IAP^{2,3}. Finally, the guanine nucleotide-sensitive agonist binding component is larger than the nucleotide-insensitive antagonist binding component^{16,17}.

In the present study we purified muscarinic receptors from the digitonin extract of porcine brain by chromatography with an affinity gel of a specific ligand, 3-(2'-aminobenzhydryloxy)tropane¹⁸. The purified protein bound 3 nmol mg^{-1} of ³H-N-methylscopolamine (³H-NMS), representing a 3,000-fold purification. The protein was iodinated and submitted to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). We found a major band with an apparent relative molecular mass (M_r) of 70,000, together with several minor bands (Fig. 1). The major band was the only band detected on rechromatography¹⁹, and proved to be the muscarinic receptor itself or its ligand-binding subunit, as it was also labelled with ³H-propylbenzylcholine mustard, an irreversible muscarinic ligand. The receptor protein purified in this way did not display GTP binding or hydrolysing activity, nor did it serve as a substrate of IAP-catalysed ADP-ribosylation.

GTP-binding proteins serving as substrates of IAP-catalysed ADP-ribosylation were purified from cholate extract of rat brain by sequential application of column chromatographic techniques using DEAE-Sephacel, Ultrogel AcA-34 and hydroxylapatite^{20,21}. The proteins freed of stimulatory guanine nucleotide regulatory protein (N_s) after passage through the final column were a mixture of two IAP substrates, as reported for bovine brain proteins^{22,23}; these were further separated on a column of DEAE-Toyopearl 650(S)²⁰. One of these heterotrimers consists of an α -, a β - and a γ - (M_r 41,000, 35,000 and ~10,000, respectively) and was used as purified N_i for the reconstitution experiments described below. No ligand for muscarinic receptors bound specifically to this protein.

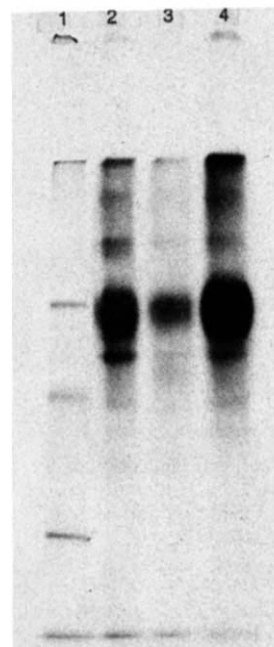


Fig. 1 SDS-PAGE of iodinated receptor preparations used for reconstitution studies. Receptor preparations obtained in three independent preparations are in lanes 2-4. Lane 1, relative molecular mass marker proteins (bovine serum albumin, 67,000; ovalbumin, 45,000; and carbonic anhydrase, 30,000).

Methods. About 900 ml of a digitonin(1%)/cholate(0.1%) extract of membrane preparations from porcine cerebrum were passed through the ABT-agarose column (3 × 43 cm, 300 ml). The ABT-agarose column was washed with 1.5 l 0.2 M NaCl/20 mM K-phosphate buffer (pH 7.0)/0.1% digitonin, then a small column of hydroxylapatite (1 ml, 0.8 × 2 cm) was connected to one end of the ABT-agarose column. Muscarinic receptors were eluted from the ABT-agarose column with buffer containing 0.1 mM atropine and allowed to bind to the hydroxylapatite. After washing the hydroxylapatite column with 0.15 M K-phosphate buffer/0.1% digitonin, muscarinic receptors were eluted with 0.5 M K-phosphate buffer/0.1% digitonin. The preparation contained 265 pmol of muscarinic receptors, with a specific activity of 3,060 pmol mg^{-1} of protein in a volume of 6 ml. The amounts of muscarinic receptors and proteins were assessed by binding of ³H-NMS and with fluorescamine³³, respectively. The purified preparation was radioiodinated by the chloramine T method³⁴ and electrophoresed by the method of Laemmli³⁵, followed by autoradiography.

The muscarinic receptor and N_i proteins thus purified were reconstituted into phospholipid vesicles essentially by the method used for β -adrenergic receptors and N_s ²⁴. A mixture of these proteins with the crude brain phospholipid fraction and phosphatidylcholine in detergents, was submitted to gel filtration to obtain detergent-free phospholipid vesicles in the void-volume fraction. About 80% of the ³H-NMS-binding activity of the receptor protein and 50% of the GTPase activity of N_i was recovered in the vesicles. The fraction of the protein-bound ³H-NMS trapped by a membrane filter (Toyo Roshi TM2) increased from 12 to 43% on reconstitution, reflecting incorporation of the protein into the reconstituted vesicles.

N_i displayed Mg^{2+} -dependent GTPase activity both in phospholipid vesicles (Fig. 2) and in 0.1% Lubrol solution²⁰. Carbachol had no effect unless the receptor protein was also reconstituted into the N_i -containing vesicles; it increased the GTPase activity of N_i at all the concentrations of Mg^{2+} studied (0.15–15 mM). The degree of increase caused by the agonist was larger at lower concentrations of Mg^{2+} ; the affinity of N_i for Mg^{2+} , an activator of the GTPase, was enhanced by stimulation of muscarinic receptors in the reconstituted vesicles (Fig. 2).

Carbachol increased GTPase activity in the receptor- N_i vesicles in a concentration-dependent manner (Fig. 3): the half-maximal effective concentration (EC_{50}) was 3.6 μ M in the presence of 2.8 mM $MgCl_2$. This value was virtually the same as the

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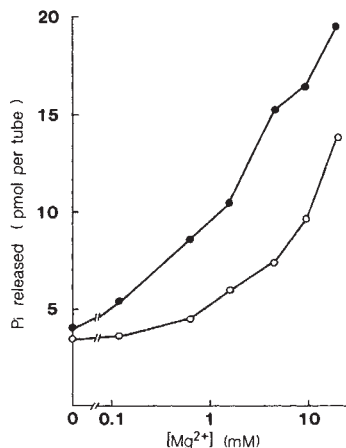


Fig. 2 Magnesium-dependent GTPase activity in receptor- N_i vesicles with or without carbachol. Concentration of free Mg^{2+} is shown on the abscissa. The incubation medium was either further supplemented with 0.1 mM carbachol (●) or contained no carbachol (○).

Methods. N_i (90 μ g or 1 nmol) in 0.7 ml (pH 8.0) of buffer containing 35 mM HEPES, 0.55 mM EDTA, 1 mM dithiothreitol (DTT), 50 mM NaCl, 125 mM K-phosphate buffer, 0.75 mM $MgCl_2$ and 0.05% sodium cholate, was mixed with muscarinic receptor (1.2 μ g or 16 pmol) in 0.7 ml 0.5 M K-phosphate buffer, 0.07% digitonin, 0.7 ml of 0.5 mg ml^{-1} dimyristoyl phosphatidylcholine, 1 mg ml^{-1} brain extract (Folchi Fraction I, Sigma), 3.6 mg ml^{-1} sodium deoxycholate and 0.4 mg ml^{-1} sodium cholate, and reconstituted by gel filtration through a Sephadex column (1.2 $\times$ 25 cm) according to Brandt *et al.*³⁶. The column was prewashed and eluted with 20 mM HEPES, 1 mM EDTA, 1 mM DTT, 0.1 M NaCl and 1.5 mM $MgCl_2$. Vesicles (20 μ l per tube) prepared by this method were incubated at 30 °C for 30 min with 0.5 μ M [γ -³²P]GTP, 15 mM HEPES, 0.75 mM EDTA, 0.75 mM DTT and 75 mM NaCl/ $MgCl_2$ in a total volume of 0.1 ml per tube.

EC_{50} (3.8 μ M) for the carbachol-induced increase in GTPase activity of rat striatal membranes²⁵. The effect of carbachol was antagonized in a concentration-dependent manner by the simultaneous addition of atropine (Fig. 3). The dissociation constant for the atropine/receptor complex was calculated to be 7 nM from the data in Fig. 3 by assuming that atropine competed with carbachol for the same sites on the receptor molecules responsible for increasing the GTPase activity of coupled N_i . This value was virtually the same as the apparent dissociation constant (7.8 nM) for atropine-induced displacement of ³H-NMS from the receptor proteins in a detergent solution¹⁹.

We also determined the potency of carbachol in displacing ³H-NMS bound to the reconstituted vesicles. The apparent dissociation constant (180 μ M) obtained was much larger than the EC_{50} (3.6 μ M) for the GTPase activity. Presumably, agonists bind with a high affinity to a small fraction of receptor proteins that are coupled to N_i , and bind with a low affinity to the residual bulk of uncoupled receptors. It is reasonable to assume that the affinity of muscarinic receptors for agonists is enhanced by their coupling to GTP-binding proteins such as N_i ¹²⁻¹⁵.

N_i purified from rat brain was ADP-ribosylated when incubated with the A-protomer^{5,6} of IAP, the active component of this A-B toxin⁴, in the presence of NAD²⁰. The ADP-ribosylated N_i exhibited the same Mg^{2+} -dependent GTPase activity as did the native (non-ADP-ribosylated) N_i in Lubrol solution or reconstituted phospholipid vesicles. There was no increase in GTPase activity, however, when carbachol was added to the vesicles into which the ADP-ribosylated N_i , instead of the native N_i , was reconstituted together with muscarinic receptors (data not shown). The direct ADP-ribosylation of N_i in the reconstituted vesicles was then achieved in the same experimental conditions as in Figs 2 and 3 by further adding 0.6 mM NAD alone (control) or NAD plus 0.2 μ g of the A-protomer of IAP (ADP-ribosylated) to the GTPase assay mixture. The assay time was extended to 60 min to allow ADP-ribosylation to proceed in the vesicles. The addition of 0.1, 1, 10, 100 and

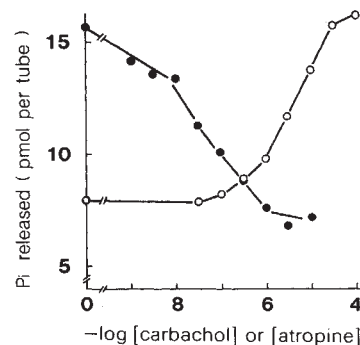


Fig. 3 Concentration-dependent increase of GTPase activity by carbachol in the absence of atropine (○) and antagonism by atropine with 33 μ M carbachol (●). Vesicles containing N_i and muscarinic receptor were prepared and incubated in a medium supplemented with 2.8 mM $MgCl_2$ as described in Fig. 2 legend.

1,000 μ M carbachol produced increases of 0, 10, 48, 86 and 112%, respectively in GTPase activity of the control vesicles (supplemented with NAD alone) and increases of 0, 0, 12, 27 and 41%, respectively, in ADP-ribosylated vesicles (NAD plus the A-protomer of IAP). The A-protomer had no effect in the absence of NAD. Thus, ADP-ribosylation by IAP interfered with the ability of N_i to couple to muscarinic receptors in the reconstituted vesicles.

In conclusion, muscarinic receptors purified from porcine brain are coupled to N_i , the IAP substrate, in phospholipid vesicles in such a manner that stimulation of the receptors by agonists gives rise to increases of the GTPase activity, or of the affinity for Mg^{2+} , of this GTP-binding protein. Subdivision of muscarinic receptors has been proposed based on tissue and agonist specificities²⁶. Two subtypes of muscarinic receptors in embryonic chick heart cells have been reported, one linked to adenylate cyclase inhibition and the other to phosphoinositide breakdown²⁷. In addition, a third type of muscarinic receptors in human astrocytoma has been reported^{28,29} which is coupled to phosphoinositide metabolism, rather than adenylate cyclase, via a GTP-binding protein that did not serve as the substrate of IAP-catalysed ADP-ribosylation, although receptor-linked activation of phospholipid metabolism and Ca^{2+} mobilization was mediated by an IAP substrate in guinea pig neutrophils³⁰ and rat mast cells^{31,32}. It remains to be shown whether the rat brain N_i coupled to muscarinic receptors is responsible for inhibition of adenylate cyclase or activation of phospholipase C under physiological conditions. Direct evidence for the receptor subtypes that interact with different effectors, and molecular characterization of these interactions should be obtained by further studies using reconstitution of purified proteins.

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Primary structure of the α -subunit of *Torpedo californica* ($\text{Na}^+ + \text{K}^+$)ATPase deduced from cDNA sequence

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Tryptic peptides from the purified *T. californica* ($\text{Na}^+ + \text{K}^+$)ATPase were isolated, analysed for amino-acid sequence and five peptide sequences determined (Fig. 1). A library of complementary DNA clones derived from poly(A)⁺ RNA from an electric organ of *T. californica* was then screened by hybridization with a mixture of synthetic oligodeoxynucleotides, which represented all possible cDNA sequences corresponding to part of the amino-acid sequence determined for one of the tryptic peptides (Fig. 1e). An upstream fragment of the cDNA insert of a clone thus isolated was used as probe to screen the cDNA library again, and a third screening was carried out with an upstream fragment of the cDNA insert of a clone that resulted from the second screening. Details of the cloning are given in the Fig. 2 legend.

Figure 2 shows the 3,365-nucleotide sequence (excluding the poly(dA) tract) of the cDNA encoding the α -subunit of *T. californica* ($\text{Na}^+ + \text{K}^+$)ATPase. Because the mRNA encoding this polypeptide has a size of ~4,000 nucleotides as estimated by blot hybridization analysis (data not shown), a 5'-terminal sequence of ~500 nucleotides is apparently missing from the sequence shown in Fig. 2. All of the five partial amino-acid sequences determined by peptide analysis (Fig. 1) were found to be encoded by the cDNA sequence in the same reading frame (amino-acid residues 228-240, 431-438, 535-550, 671-690 and 1,011-1,022). By using this reading frame, the amino-acid sequence of the cryptic portion of the ($\text{Na}^+ + \text{K}^+$)ATPase α -subunit was deduced (Fig. 2). The translational initiation site was assigned to the methionine codon at nucleotide residues 1-3 because this is the first in-frame ATG triplet that appears downstream of the nonsense codon TAA (nucleotide residues -39 to -37). Codon 1,022 specifying tyrosine is followed by a translational termination codon (TAG). Thus, we conclude that the α -subunit of *T. californica* ($\text{Na}^+ + \text{K}^+$)ATPase is composed of 1,022 amino-acid residues (including the initiating methionine) and has a calculated M_r of 112,424. This polypeptide does

Sodium- and potassium-dependent ATPase [($\text{Na}^+ + \text{K}^+$)ATPase], which is responsible for the active transport of Na^+ and K^+ , is distributed universally among animal cell membranes and consists of two types of subunits, α and β (refs 1-4). The larger α -subunit with a relative molecular mass (M_r) of 84,000-120,000 is thought to have the catalytic role. We have now cloned and sequenced DNA complementary to the *Torpedo californica* electroplax messenger RNA encoding the α -subunit of ($\text{Na}^+ + \text{K}^+$)ATPase and have deduced the complete amino-acid sequence of the polypeptide. Some structural features of the α -subunit molecule related to the function of this active-transport protein are discussed.

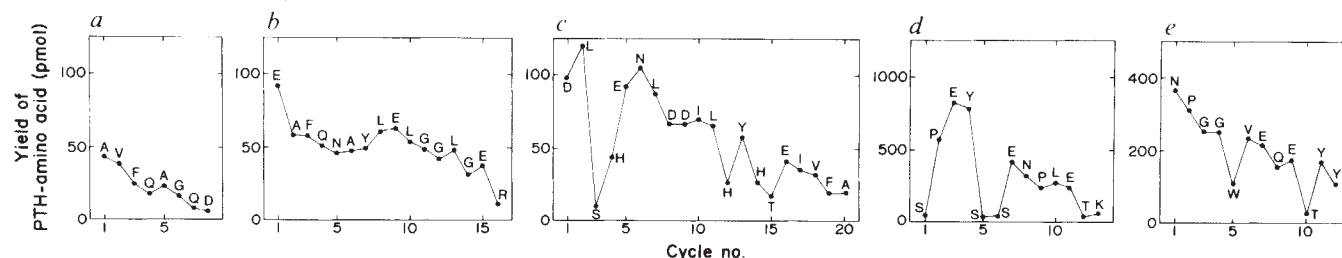
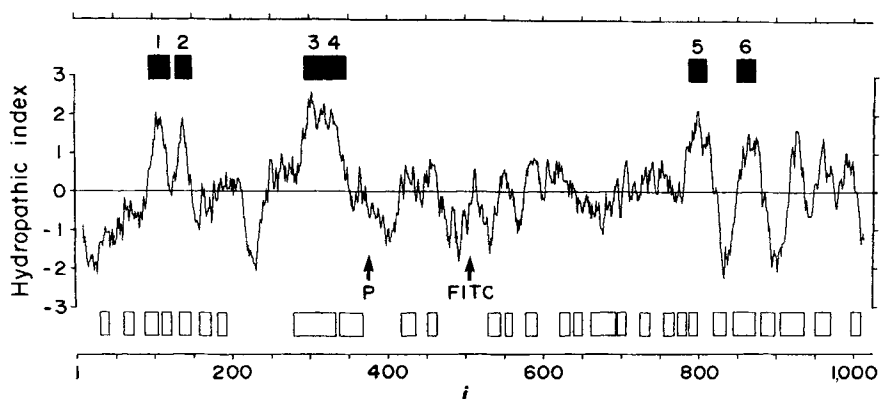


Fig. 1 Sequence analysis of peptides from the α -subunit of *T. californica* ($\text{Na}^+ + \text{K}^+$)ATPase. Fractions Ia (a), IIa (b), IIIa (c), IVa (d) and Va (e) were subjected to amino-acid sequence analysis with a gas-phase sequencer²⁶ (model 470 A, Applied Biosystems).

Methods. The microsomal fraction (673 mg of protein) derived from ~200 g of *T. californica* electric organ (Pacific Bio-Marine Laboratories) was treated with SDS²⁷ in the presence of 3 mM ATP and 30% (w/v) glycerol and centrifuged in the discontinuous gradient of 30% (w/v) and 42% (w/v) sucrose. The material at the interface was collected and treated with 3 mg ml⁻¹ octaethylene glycol mono-*n*-dodecyl ether⁹ at a protein concentration of 1.5 mg ml⁻¹ and then centrifuged. The resulting supernatant contained 81 mg of purified ($\text{Na}^+ + \text{K}^+$)ATPase. The enzyme was treated with 1% SDS and fractionated by gel filtration on a Biogel A-1.5m column²⁸ (2.5 × 100 cm). The fraction consisting mainly of the α -subunit was subjected to chromatography on a concanavalin A-Sepharose column²⁹ (1.2 × 11 cm) followed by gel filtration on the same Biogel A-1.5m column to yield 1.2 mg of purified α -subunit. This was dialysed for two days against 10 mM sodium phosphate buffer, pH 7.2, containing 6 M urea, in which particles of Dowex AG1-X2 resin were suspended for efficient removal of SDS, and was then dialysed for 20 h against 0.1 M ammonium bicarbonate, pH 8.0 or 0.2 M ammonium acetate, pH 8.0. The dialysed material was concentrated and treated with TPCK-treated trypsin (Worthington) at room temperature for 16 h in the absence or presence of 10 mM CaCl₂; the α -subunit/trypsin ratio was 50. A further identical aliquot of TPCK-treated trypsin was added, and the incubation was continued for additional 8 h. The tryptic digest was directly loaded onto a Hi-Pore C₄ column (4.6 × 250 mm; Bio-Rad) equilibrated with 0.1% trifluoroacetic acid, and a linear gradient of 0-50% (over 50 min) or 0-30% (over 60 min) acetonitrile was applied at a flow rate of 1 ml min⁻¹. Five fractions were collected and rechromatographed on the same column to yield fractions Ia-Va. Phenylthiohydantoin (PTH)-amino acids were analysed by HPLC at 40 °C using a Senshu-Pak column (4.6 × 300 mm) with a three-solvent gradient (acetonitrile, water and 40 mM sodium acetate buffer, pH 4.7) at a flow rate of 1 ml min⁻¹. The yield of PTH-amino acids at each cycle of Edman degradation is shown; the one-letter amino acid notation is used.

Fig. 3 Hydropathy profile and predicted secondary structures of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ α -subunit. The averaged hydropathic index of a nonadecapeptide composed of amino-acid residues $i-9$ to $i+9$ is plotted against i , where i represents amino acid number; the hydropathy indices of individual amino acids are taken from ref. 12. The positions of the predicted structures of α -helix and/or β -sheet¹³ that have a length of ≥ 10 residues are shown by open boxes, and those of the putative transmembrane segments M1-M6 by closed boxes. The phosphorylation and FITC-reactive sites are indicated.



not possess a hydrophobic amino-terminal sequence characteristic of the signal peptide⁵.

The sequence of amino-acid residues 2-17 (with a gap of residues 4-7) is homologous with the amino-terminal sequence of the mammalian^{6,7} and avian $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ α -subunit⁸, and the sequence of residues 2-47 (with gaps of residues 5-19, 22 and 27-30) with that of the *Artemia salina* counterpart⁹. Furthermore, some of the partial sequences of the lamb kidney $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ α -subunit, determined by analysis of tryptic peptides¹⁰, have homologous counterparts in the *Torpedo* sequence (for example, residues 502-512, 596-606 and 647-657). Five potential *N*-glycosylation sites¹¹ (residues 215, 405, 483, 637 and 719) are found in the *Torpedo* α -subunit sequence, but all of them are assigned to the cytoplasmic side of the membrane (see below).

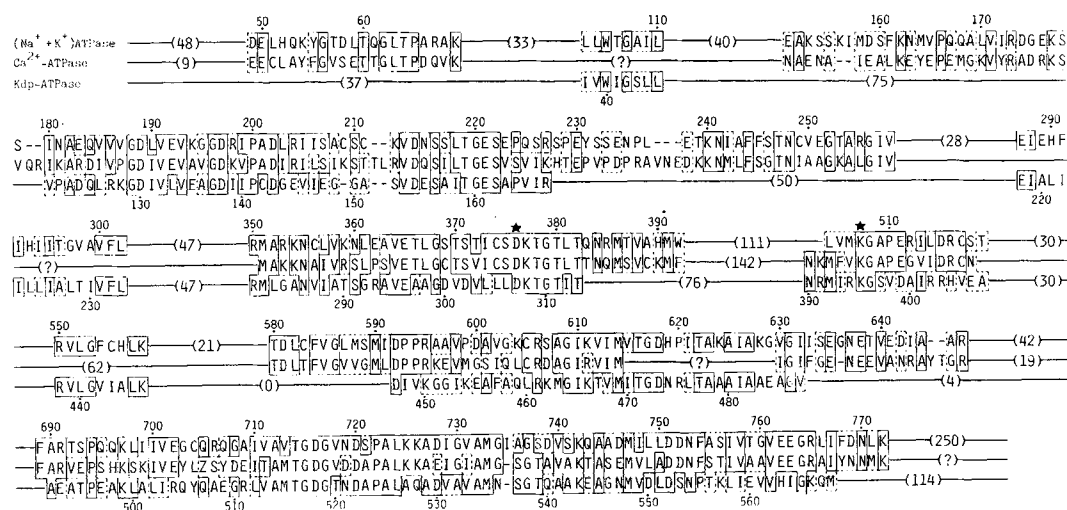
Analysis of the amino-acid sequence of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ α -subunit for local hydropathy¹² and predicted secondary structure¹³ (Fig. 3) suggests the presence of at least six transmembrane segments forming presumably α -helical structures, that is, M1 (residues 95-122), M2 (129-150), M3 (294-318), M4 (320-348), M5 (786-809) and M6 (848-873). These hydrophobic segments comprise a continuous stretch of 22-29 uncharged residues including many nonpolar residues, except that segment M4 contains a glutamic acid residue (residue 334) in the middle, which is flanked by proline and glycine. If segment M5 or M6 is assumed to extend over a wider range (residues 774-823 and 848-885, respectively), the possibility

remains that one or both of these segments span the membrane twice in a hairpin shape. If this was the case, segments M5 and M6 would also contain three and two acidic residues, respectively, as sole charged residues.

It is possible that the transmembrane segments take part in the cation-conducting pathway and that the acidic residues within these segments are involved in the transport of cations. Of interest in this context is that $(\text{Na}^+ + \text{K}^+)\text{ATPase}$, in its normal catalytic cycle, passes through states in which cations are occluded within the enzyme molecule so that they are not exchangeable^{14,15}. Three additional hydrophobic segments comprising exclusively uncharged residues (residues 919-931, 961-973 and 984-1,000) are present near the carboxy-terminus (Fig. 3). They seem to be too short to span the membrane, but the possibility remains that some of them traverse the membrane if a longer stretch is assumed. They may be associated with the membrane or may be involved in hydrophobic inter- or intra-subunit interaction.

The sequence surrounding the aspartic acid residue that is phosphorylated by ATP¹⁶ (Cys-Ser-Asp-Lys) is located at residues 374-377. The site reactive with fluorescein 5'-isothiocyanate (FITC)^{17,18} (His-Leu-Leu-Val-Met-Lys-Gly-Ala-Pro-Glu-Arg), which is thought to be at or near the ATP binding site, is positioned at residues 502-512, except that the amino-terminal histidine residue is replaced by tyrosine. Because these functional regions reside on the cytoplasmic side of the membrane, the transmembrane topology of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$

Fig. 4 Alignment of the amino-acid sequences of the *T. californica* $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ α -subunit (top), rabbit skeletal sarcolemmal Ca^{2+} -ATPase (middle) and the KdpB protein of *E. coli* Kdp-ATPase (bottom). The sequences of the Ca^{2+} -ATPase and the KdpB protein have been taken from refs 24 and 25, respectively. The one-letter amino-acid notation is used. Sets of identical residues are enclosed with solid lines and sets of conservative residues by dotted lines; conservative substitutions are defined as pairs of residues belonging to one of the following groups: S, T, P, A and G; N, D, E, Q and Z; H, R and K; M, I, L and V; F, Y and W (ref. 37). Gaps (—) have been inserted to achieve maximum homology. The non-homologous segments are shown by lines together with the sum of residues present in each segment given in parentheses. The residues of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ α -subunit and the KdpB protein are numbered beginning with the initiating methionine of the respective polypeptides. The residues of the Ca^{2+} -ATPase, whose numbers (see ref. 24) are not shown, are as follows (from the amino- to the carboxy-terminus): sequence 1 (S1), 10-30; S2, 3-115; S3, 1-42; S3, 185-201; S3, 264-298; S4, 1-18; S4, 38-120. The aspartic acid residue that is phosphorylated and the lysine residue reactive with FITC are marked with asterisks. For evaluating homology (see text), only the segments comprising residues 180-227, 351-382, 504-518, 592-614, 630-632 and 690-767 of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ have been taken into account; gaps have been counted as one substitution regardless of their length, and the position including Z has been excluded from the calculation for all pairs.



as pairs of residues belonging to one of the following groups: S, T, P, A and G; N, D, E, Q and Z; H, R and K; M, I, L and V; F, Y and W (ref. 37). Gaps (—) have been inserted to achieve maximum homology. The non-homologous segments are shown by lines together with the sum of residues present in each segment given in parentheses. The residues of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ α -subunit and the KdpB protein are numbered beginning with the initiating methionine of the respective polypeptides. The residues of the Ca^{2+} -ATPase, whose numbers (see ref. 24) are not shown, are as follows (from the amino- to the carboxy-terminus): sequence 1 (S1), 10-30; S2, 3-115; S3, 1-42; S3, 185-201; S3, 264-298; S4, 1-18; S4, 38-120. The aspartic acid residue that is phosphorylated and the lysine residue reactive with FITC are marked with asterisks. For evaluating homology (see text), only the segments comprising residues 180-227, 351-382, 504-518, 592-614, 630-632 and 690-767 of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ have been taken into account; gaps have been counted as one substitution regardless of their length, and the position including Z has been excluded from the calculation for all pairs.

α -subunit molecule is assigned such that the amino-terminal region, the regions between segments M2 and M3 and between segments M4 and M5 and the carboxy-terminal region are located on the cytoplasmic side (assuming the presence of six transmembrane segments). Thus, the regions between segments M1 and M2, between segments M3 and M4 and between segments M5 and M6 are assigned to the extracellular side of the membrane, where the ouabain binding site is situated. The transmembrane topology assigned is generally consistent with the model proposed on the basis of specific covalent labelling combined with proteolysis¹⁹⁻²¹. Furthermore, our assignment generally supports the asymmetric partition of the α -subunit molecule across the membrane, favouring the cytoplasmic region^{22,23}.

Figure 4 shows the alignment of the homologous amino-acid sequences of the *T. californica* ($\text{Na}^+ + \text{K}^+$)ATPase α -subunit, rabbit skeletal sarcoplasmic reticulum Ca^{2+} -ATPase²⁴ and the KdpB protein of *Escherichia coli* Kdp-ATPase²⁵ (more than half of the sequence of the Ca^{2+} -ATPase has been determined by protein analysis²⁴). The degree of homology for the total segments where sequences of all the three polypeptides can be aligned is 58, 37 and 36% for the pairs ($\text{Na}^+ + \text{K}^+$)ATPase/ Ca^{2+} -ATPase, ($\text{Na}^+ + \text{K}^+$)ATPase/Kdp-ATPase and Ca^{2+} -ATPase/Kdp-ATPase, respectively. This suggests that the three polypeptides evolved from a common ancestor. The regions conserved in all the three polypeptides, including the phosphorylation and FITC binding sites, may be involved in the basic energy transduction process common to these ion transport ATPases rather than in their ion selectivity, which is different for each enzyme.

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Voltage-dependent ATP-sensitive potassium channels of skeletal muscle membrane

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It has been known for some years that skeletal muscle develops a high potassium permeability in conditions that produce rigor, where ATP concentrations are low and intracellular Ca^{2+} is high¹. It has seemed natural to attribute this high permeability to K channels that are opened by internal Ca^{2+} , especially as the presence of such channels has been demonstrated in myotubes and in the transverse tubular membrane system of adult skeletal muscle²⁻⁴. However, as we show here, the surface membrane of frog muscle contains potassium channels that open at low internal concentrations of ATP (<2 mM). ATP induces closing of these channels without being split, apparently holding the channels in one of a number of closed states. The channels have at least two open states whose dwell times are voltage-dependent. Surprisingly, we find that these may be the most common K channels of the surface membrane of skeletal muscle.

To record unitary currents through the potassium channels, we have used the patch-clamp technique⁵ on large sarcolemmal vesicles prepared by KCl loading and enzymatic treatment^{6,7}. We used recording pipettes filled with a 60 mM KCl solution in most cases, and held the membrane potential of the patch at a negative level. In these conditions, channel activity appeared only when the patch was excised from the vesicle, so that its inner surface came into contact with a 120 mM KCl solution, free of ATP. Figure 1 illustrates one such experiment, in which the patch was held at a membrane potential of -60 mV and was exposed in turn to solutions containing ATP at 0.5 and 2 mM, interspersed with exposures to ATP-free solution. The presence of ATP reduces the probability of the channels being open, P_{open} , from 0.496 ± 0.152 ($n = 16$) in ATP-free solution at -60 mV (60 mM external $[\text{K}]_0$) to 0.154 ± 0.033 ($n = 4$) in 0.5 mM ATP.

ATP closes channels without being metabolized. First, most of the patches were exposed to internal solutions free of Mg^{2+} (three of the four patches exposed to 0.5 mM ATP). Second, the non-metabolizable analogue of ATP, adenylylimidodiphosphate (AMP-PNP, 0.5 mM) was equally effective at closing channels, reducing P_{open} to 0.129 and 0.226 in two patches. (In one further patch, 0.5 mM ATP and 0.5 mM AMP-PNP both silenced all four channels that the patch contained.) However, ADP was much less effective than ATP.

The ATP-sensitive channel is selective for K^+ , as its current-voltage relation (Fig. 2c) reverses at the equilibrium potential for potassium ions (E_K). With 60 mM K^+ in the pipette, this reversal potential was -18.2 ± 0.7 mV ($n = 5$), close to the theoretical value of -17.3 mV. Reducing the pipette K^+ concentration altered the reversal potential appropriately, to -27 mV in 40 mM $[\text{K}]_0$ (one patch) and to -54 and -60 mV (two patches) in 10 mM $[\text{K}]_0$. Channel conductance was also dependent on external K^+ concentration, being reduced from 48.2 ± 1.5 pS ($n = 5$) in 60 mM K^+ to 17.8 and 18.0 pS in 10 mM K^+ . The channel is highly selective: Rb^+ does not permeate when it is substituted for K^+ in the pipette (not shown), even though it permeates several other classes of K channel.

As well as being decreased by the presence of ATP, channel opening is also affected by membrane potential, although it is not increased by Ca^{2+} , unlike the opening of Ca-activated K channels, which also have a higher unitary conductance³. We have demonstrated the voltage dependence in two ways: by holding the potential close to E_K and then stepping the potential for periods of 50 ms (Fig. 2a, b), and by recording for prolonged periods (average ~90s) at different holding potentials and subsequently measuring open and closed times (Fig. 3). The experi-

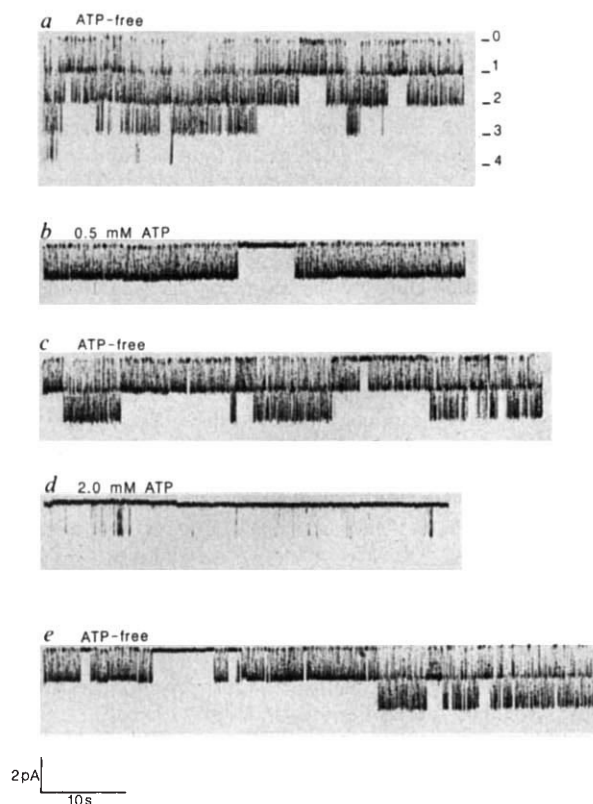


Fig. 1 Effect of ATP on K^+ -channel currents in an inside-out patch of membrane excised from a sarcolemmal vesicle formed from frog muscle⁷. The patch pipette contained 60 mM KCl, 60 mM NaCl, 1.8 mM $CaCl_2$ and 5 mM Tris-maleate, pH 7.2. The membrane potential was -60 mV throughout. The cytoplasmic face of the membrane was exposed to different ATP concentrations by moving the pipette tip between the mouths of a series of pipes down which test solutions flowed at $1-2$ ml h^{-1} (ref. 16). These solutions contained 5 mM EGTA neutralized with KOH, KCl to bring $[K^+]$ to 120 mM, 2 mM $MgCl_2$, ATP at the concentration indicated, and 10 mM HEPES buffer, pH adjusted to 7.2 with KOH. The patch was exposed to the solutions in the order *a*, *b*, *c*, *d*, *e* and we have shown the total time of recording in each case. Currents were recorded with a List electronic EPC-5 patch-clamp amplifier onto FM tape (Racal Store 4, 30 i.p.s.) and were filtered at 0.5 kHz (3 dB down, 8-pole Bessel) before display. The current levels corresponding to 0, 1, 2, 3 and 4 open channels are indicated at the right of record *a*. P_{open} was calculated as $(\sum_{j=1}^N t_j)/TN$ where t_j is the time spent at each level, j corresponding to 1, 2, ..., N channels open (where N is the maximum number of channels seen in ATP-free solution), and T is the duration of the recording. Analysis was done using a computer after the current record had been digitized (see Fig. 2 legend). In this experiment the P_{open} values were: *a*, 0.401; *b*, 0.175; *c*, 0.251; *d*, 0.004; *e*, 0.222. Mean values in ATP-free solution and in 0.5 mM ATP are given in the text. A second patch exposed to 2 mM ATP had $P_{open} = 0.012$. In two patches exposed to 0.1 mM ATP, P_{open} was 0.255 and 0.225; in the same patches in 0.02 mM ATP, P_{open} was 0.398 and 0.344.

ments involving stepping of the membrane potential show that the mean current per trace declines with time under hyperpolarization (Fig. 2*a*), indicating that hyperpolarization partly inactivates the potassium conductance. However, depolarization from -17 mV does not further activate conductance (Fig. 2*b*). We used this stepping procedure routinely to measure current-voltage relations for the channel (as in Fig. 2*c*).

The records of Figs 1 and 2*a* indicate that the channel opens and shuts rapidly. Channel openings occur in bursts separated by intervals that may be quite brief (Fig. 2*a*) or very long (Fig. 1*b*, *e*), showing that the channel has complex kinetics. Open-time histograms (Fig. 3*a-c*) are better fitted by two exponentials, suggesting two open states⁸. The presence of two components in the open time distribution is clearer at -40 mV (Fig. 3*c*) than at -60 mV (Fig. 3*a*), partly because of an increase in time

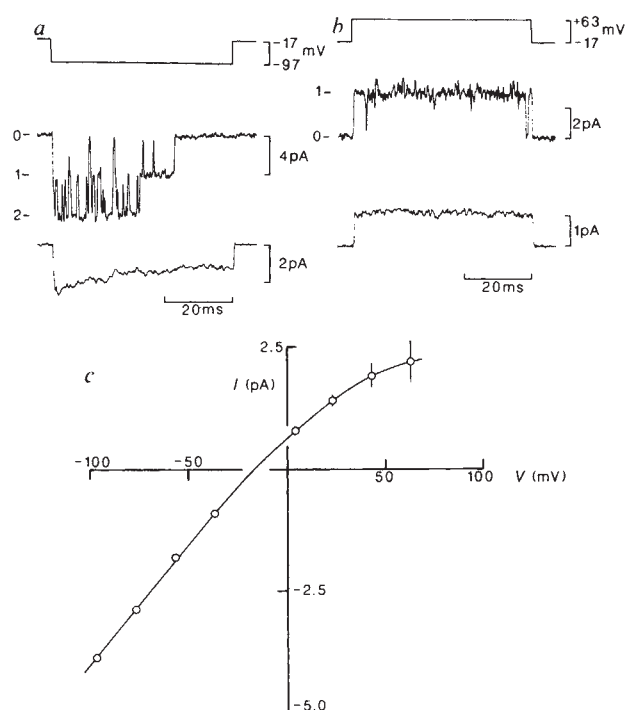


Fig. 2 *a*, Single-channel currents recorded from ATP-sensitive channels when a membrane patch was stepped for 50 ms from the holding potential of -17 mV (close to the calculated E_K) to -97 mV. The top trace shows the voltage step, the middle trace a sample current record, and the bottom trace the average of 64 such current records. The current was filtered at 2 kHz and digitized at 10 kHz (Cambridge Electronic Design 502) before analysis with a DEC PDP 11/23 computer. Capacitative and leakage currents caused by the voltage step have been removed from the current trace by analog compensation and digital subtraction⁷. For display, the number of data points in the current records was increased fourfold by interpolation using a cubic spline method¹⁷. We used this voltage-step protocol as it has been commonly used to study the 'inward rectifier' K channels of muscle¹⁸. The ATP-sensitive channels described here are not inward rectifier channels, however, as they are not open at the resting potential on intact muscle fibres and they show only a slight rectification, (*c*), rather than the extreme rectification associated with the inward rectifier. Also, their single-channel conductance is much larger than that measured for inward rectifier channels by noise analysis¹⁹. *b*, Currents recorded as in *a* but for a step in potential to $+63$ mV. Top trace, voltage; middle trace, current; bottom trace, average of 24 current records. *c*, Mean single-channel current-voltage ($I-V$) relation for the ATP-sensitive channel. Single-channel currents were recorded using 50-ms steps in membrane potential as in *a* and *b*; current amplitude was measured by fitting gaussian distributions to histograms of digitized current values⁷. The points show the mean values for four or five patches and the bars show the s.e.m. where this is larger than the symbols. The recording pipette contained 60 mM K^+ , while the solution bathing the cytoplasmic face of the patch contained 120 mM K^+ . The decrease in conductance at positive potentials was associated with an increase in the open-channel current noise, as can be seen in the middle record of *b*.

constant of the slower component with depolarization. There appear to be relatively more brief openings in the presence of ATP (Figs 1*d*, 3*b*).

Closed-time distributions (Fig. 3*d-f*) indicate the presence of at least three closed states. However, we have not recorded for sufficiently long periods to measure the length of more than a few of the very long periods of closing, which are therefore not well represented in the closed-time histograms of Fig. 3. In fact, ATP apparently increases the chance of the channel entering a closed state with a very long dwell time. ATP reduced the several levels of opening seen in multichannel patches to one or two levels of opening (see Fig. 1), indicating that several of the channels are silent throughout the 90-s recording period. The shorter closed times in Fig. 3*b* are little affected by ATP.

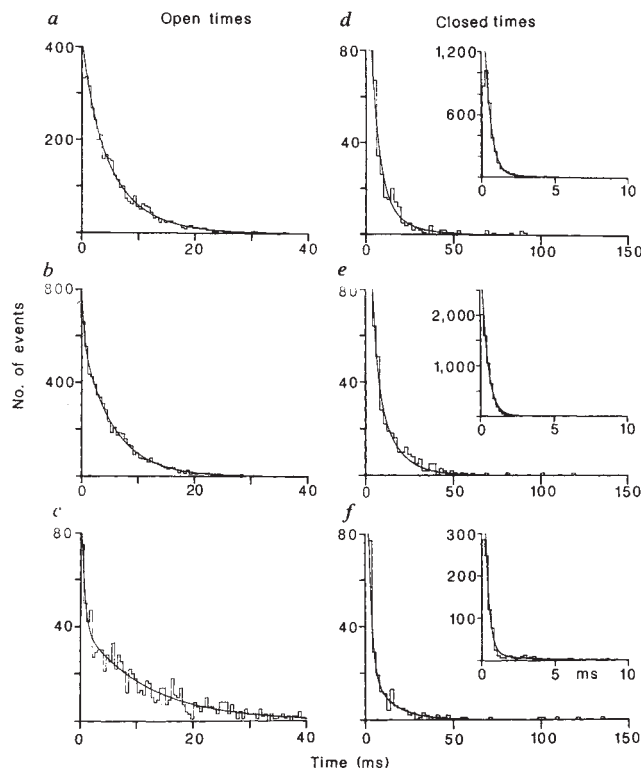


Fig. 3 Histograms of open (*a-c*) and closed (*d-f*) times for the ATP-sensitive channel, constructed from periods of recording lasting between 39 and 51 s, during which only a single level of opening was seen. The pipette contained 60 mM K^+ . *a-c*, Effects of ATP (*a, b*) and membrane potential (*a, c*) on open-time distribution. These distributions have been fitted using a least-squares algorithm to the double-exponential probability density function (p.d.f.):

$$f(t) = A \exp(-t/\tau_1) + B \exp(-t/\tau_2).$$

ATP increases the value of A , reduces τ_1 , but has little effect on τ_2 . Depolarization increases τ_2 . *a*, Open-time histogram in ATP-free solution; holding potential -60 mV. The fitted p.d.f. has $A = 0.029 \text{ ms}^{-1}$, $B = 0.168 \text{ ms}^{-1}$, $\tau_1 = 1.85 \text{ ms}$ and $\tau_2 = 5.65 \text{ ms}$. *b*, Open-time histogram at -60 mV in 0.5 mM ATP solution for the same patch as *a*. The fitted p.d.f. has $A = 0.109 \text{ ms}^{-1}$, $B = 0.172 \text{ ms}^{-1}$, $\tau_1 = 0.34 \text{ ms}$, $\tau_2 = 5.58 \text{ ms}$. *c*, Open-time histogram at -40 mV in ATP-free solution. The fitted p.d.f. has $A = 0.118 \text{ ms}^{-1}$, $B = 0.081 \text{ ms}^{-1}$, $\tau_1 = 0.59 \text{ ms}$, $\tau_2 = 11.56 \text{ ms}$. *d-f*, Effects of ATP (*d, e*) and membrane potential (*d, f*) on closed-time distribution. These distributions have been fitted with three exponentials. The two slowest were fitted to a histogram formed with 2-ms bins and the fastest was fitted after the histogram had been re-formed using 0.2-ms bins (insets). The fitted p.d.f. is given by

$$f(t) = A \exp(-t/\tau_1) + B \exp(-t/\tau_2) + C \exp(-t/\tau_3)$$

ATP has little effect on the distribution measured, but appears to place channels in a closed state too long-lived to contribute significantly to the histograms shown. *d*, Closed-time histograms in the same conditions and for the same patch as in *a*. The fitted p.d.f. has $A = 2.231 \text{ ms}^{-1}$, $B = 0.012 \text{ ms}^{-1}$, $C = 0.002 \text{ ms}^{-1}$, $\tau_1 = 0.41 \text{ ms}$, $\tau_2 = 4.56 \text{ ms}$, $\tau_3 = 14.23 \text{ ms}$. *e*, Closed-time histograms in the same conditions as in *b*. The fitted p.d.f. has $A = 2.331 \text{ ms}^{-1}$, $B = 0.011 \text{ ms}^{-1}$, $C = 0.004 \text{ ms}^{-1}$, $\tau_1 = 0.40 \text{ ms}$, $\tau_2 = 2.64 \text{ ms}$, $\tau_3 = 11.07 \text{ ms}$. *f*, Closed-time histograms in the same conditions and for the same patch as in *c*. The fitted p.d.f. has $A = 2.997 \text{ ms}^{-1}$, $B = 0.066 \text{ ms}^{-1}$, $C = 0.008 \text{ ms}^{-1}$, $\tau_1 = 0.26 \text{ ms}$, $\tau_2 = 1.8 \text{ ms}$, $\tau_3 = 12.41 \text{ ms}$.

The ATP-sensitive channels are very common in skeletal muscle. The patches that we have analysed in detail contained an average of 3.6 ± 0.4 channels ($n = 16$). Several other patches were not analysed because of the very high number of channels (occasionally as many as 10 or more) that were active. As our patch electrodes had tips with openings of only $0.3\text{--}0.4 \mu\text{m}$ diameter (as measured with a scanning electron microscope), a high channel density is indicated—as high as or higher than that of 'delayed rectifier' K channels in the same preparation⁹.

However, while the role of delayed rectifier channels is well

known (to repolarize the action potential), that of ATP-sensitive channels is less clear, particularly since the ATP concentration in frog muscle is high (4.9 mM)^{10,11} and is well buffered by creatine phosphate and creatine kinase. We find that the channels are closed in patches formed on resting intact muscle, but anticipate that channels will open under rigor conditions, contributing to the high potassium permeability then found¹. Nevertheless, it seems likely that channels that are present at such high density will have an important physiological role. While we point out that the regulating effect of ATP occurs at concentrations much closer to the physiological range than has been reported previously for ATP-sensitive channels of heart muscle¹² and of B cells of pancreatic islets^{13,14}, it is possible that the sensitivity to ATP in intact membranes is different from that found in excised patches or that some other metabolic constituent of sarcoplasm contributes to their control.

In man, the severest exercise may reduce the concentration of ATP in muscle by 45% (ref. 15). As we have found that open times for the ATP-sensitive channel also increase with depolarization, the channel may play a part in the regulation of K conductance during muscle activity. Alternatively, as the channel is present in surface membrane of muscle (and in non-contractile cells^{13,14}), its role may be primarily to sense submembrane, rather than myofibrillar, levels of ATP or other metabolites.

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I-J as an idiom of the recognition component of antigen-specific suppressor T-cell factor

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The I-J determinant of membrane glycoprotein is known to be expressed exclusively on suppressor T cells (Ts), which have a crucial role in the regulation of immune responses¹. I-J also comprises part of the soluble factor (TsF) with suppressor activity which is secreted from Ts. Gene-mapping experiments have indicated that the I-J gene lies between the I-A and I-E subregions of the mouse major histocompatibility complex (MHC)^{2,3} and is defined by the H-2 congenic pair, that is, B10.A(3R) and B10.A(5R)⁴. In fact, antibodies raised in the reciprocal combina-

tions of B10.A(3R) and B10.A(5R) define the $I-J^b$ and $I-J^k$ alleles, and are able to detect the I-J determinants on Ts and TsF. Biochemical and functional analyses⁵⁻¹⁰, using I-J-positive Ts clones and hybridomas, have demonstrated that monoclonal anti-I-J antibodies precipitate $I-J^k$ or $I-J^b$ with a relative molecular mass of 25,000–28,000 (25–28K) and that the $I-J^+$ molecule mediates the restriction specificity of TsF in association with an antigen-binding protein (45K). However, molecular genetic studies on the $I-J$ gene¹¹⁻¹³ reveal no genetic difference between B10.A(3R) and B10.A(5R) and also that there is no room to accommodate a gene encoding I-J in the expected I region. These discrepancies between the molecular genetic and serological/functional data require explanation. Here we demonstrate that Ts and TsF expressing I-J of the host type were produced by fully allogeneic bone marrow cells of donor origin in chimaeric mice, when the chimaeras received the host antigen-presenting cells (APC) at the time of immunization. The results show that APC are necessary for the activation and clonal expansion of Ts and also support the notion that I-J is an idiotype determinant of the recognition component of Ts and TsF.

To analyse the nature of I-J, chimaeric mice were prepared as described elsewhere¹⁴. C57BL/6($H-2^b$) or C3H($H-2^k$) mice were irradiated with 10.63 Gy and reconstituted with T-cell-depleted allogeneic bone marrow cells from C3H($H-2^k$), C57BL/6($H-2^b$), B10.A(3R)($H-2^{i3}$) or B10.A(5R)($H-2^{i5}$) mice. The chimaeras were kept for 4 months, then immunized with keyhole limpet haemocyanin (KLH). Primed thymocytes and spleen cells were tested for their H-2 phenotypes before preparation of TsF and were shown to possess the H-2 type of donor origin (Table 1). KLH-specific suppressor T-cell factor (KLH-TsF) was prepared by freezing and thawing of thymocytes from KLH-primed chimaeric mice, as described elsewhere³, and tested for its effects on the *in vitro* secondary anti-dinitrophenyl (DNP) IgG plaque-forming cell (PFC) responses of $H-2^k$ and $H-2^b$ mice. As shown in Table 2, KLH-TsF from the C3H→C57BL/6 chimaera, like C3H TsF, suppressed the C3H but not the C57BL/6 response in a KLH-specific manner, because only the C3H response to DNP-KLH, but not to DNP-ovalbumin (OVA), was suppressed. Similarly, the C57BL/6→C3H TsF specifically suppressed the response of C57BL/6 but not that of C3H. Therefore, KLH-TsF from chimaeric mice expresses the restricting specificity of the donor type. These results indicate that the chimaeric mice produce TsF bearing I-J of the donor type, as the restriction specificity is mediated by the $I-J^+$ molecule^{7,8} and because of the fact that the I-J phenotype of TsF must be the same as that of the responding cells^{7,8,15}. A similar conclusion was reached for chimaeras using B10.A(3R) or B10.A(5R) as the donor (Table 2). B10.A(3R)→C3H and B10.A(3R)→C57BL/6 produced $H-2^b$ -restricted TsF whereas TsF from either B10.A(5R)→C3H or B10.A(5R)→C57BL/6 suppressed only the $H-2^k$ responses. Thus, KLH-TsF from chimaeras using B10.A(3R) or B10.A(5R) is the same as KLH-TsF from conventional B10.A(3R) or B10.A(5R) mice, which produce $I-J^b$ -TsF or $I-J^k$ -TsF, respectively⁷.

The results in Table 2 show that the host-specific restriction specificity of TsF is not acquired in the chimaera situation and indicate that the I-J phenotype cannot be changed, thus I-J seems to be an MHC-controlled product. It is, however, possible that only the donor-restricted but not host-specific Ts are allowed to expand after antigenic stimulation because there is no APC of the host type present and most APC are replaced by those of donor origin in the 4-month chimaera^{14,16}. To test this possibility, the chimaeric mice were given APC of the host type at the time of immunization with KLH. As shown in Table 2, KLH-TsF from either the C3H→C57BL/6 or C57BL/6→C3H chimaera that received the host APC specifically suppressed the responses of both C57BL/6 and C3H but not those of a third party, such as BALB/c mice, despite the fact that these chimaeric thymocytes expressed H-2 of donor type alone. It is clear, therefore, that fully allogeneic chimaeras (subjected to irradiation of the bone marrow) can generate both donor and host H-2-restricted KLH-TsF. Whether the former or the latter type of TsF is

Table 1 H-2 typing of thymocytes from fully allogeneic chimaeras

Chimaeras	% Cytotoxicity with antibodies against H-2D ^b	H-2D ^d	H-2K ^b	H-2K ^k
C3H→C57BL/6	<10%	—	—	>95%
C57BL/6→C3H	>95%	—	—	<10%
C3H→C57BL/6 with C57BL/6 APC	<10%	—	—	>95%
C57BL/6→C3H with C3H APC	>95%	—	—	<10%
B10.A(3R)→C57BL/6	<10%	>95%	—	—
B10.A(3R)→C3H	—	—	>95%	<10%
B10.A(5R)→C57BL/6	<10%	>95%	—	—
B10.A(5R)→C3H	—	—	>95%	<10%

Fully allogeneic chimaeric mice were prepared as described previously¹⁴. Briefly, C57BL/6($H-2^b$) or C3H($H-2^k$) mice were irradiated with 10.63 Gy of ¹³⁷Cs γ -rays, and reconstituted 24 h later with 10⁷ T cell-depleted allogeneic bone marrow cells from C3H($H-2^k$), C57BL/6($H-2^b$), B10.A(3R)($H-2^{i3}$) or B10.A(5R)($H-2^{i5}$) pretreated with monoclonal anti-Thy-1.2 antibody (F7D5; Olac Ltd, Bicester, UK) plus complement. The mice were maintained for 4 months in pathogen-free conditions and then used for experiments. The chimaeras (with or without addition of APC of the host type) were immunized with 200 μ g of keyhole limpet haemocyanin (KLH) after a 2-week interval as described elsewhere³. Primed spleen cells and thymocytes from the chimaeras were tested for their H-2 phenotypes in a dye exclusion cytotoxicity assay using anti-H-2D^d antiserum (B10A.(2R) anti-B10.A) and various monoclonal anti-H-2 antibodies, that is anti-H-2D^b (H141-30; BALB/c anti-C57BL/6), anti-H-2K^b (K9-178.9; (C3H×A)_F₁ anti-70.Z/3) and anti-H-2K^k (H100-5.28; BALB/c anti-CBA) (given by Dr. G. J. Hammerling) before preparation of TsF. For the cytotoxicity test, spleen cells or thymocytes (10⁵) from the chimaeras were resuspended in Dulbecco's modified minimal essential medium enriched with 2% fetal calf serum (FCS) and were incubated with 20 μ g of a 1:10 dilution of anti-H-2 antiserum and monoclonal anti-H-2 antibodies at a final concentration of 1:1,000 for 15 min at room temperature. The cells were washed twice, then reacted with 20 μ l of a 1:10 dilution of rabbit complement at 37 °C for 30 min. Dead and viable cells were counted under the light microscope by Trypan blue dye exclusion.

generated depends solely on the H-2 type of the APC available at the time of antigen stimulation. The above results also show that APC are necessary for the activation and clonal expansion of Ts. These findings support the data of Takaoki *et al.*¹⁷ and Lowy *et al.*¹⁸, who demonstrated that accessory cells are involved at various stages in suppressor cell interactions.

Two further experiments were done to obtain direct evidence that I-J of the host type is indeed generated from the donor cell type. The first experiment was designed to investigate the H-2 phenotype of the cells producing TsF having host or donor restriction specificity. Thymocytes from C57BL/6→C3H chimaeras that received C3H APC were treated with antibodies against H-2 products of the host or donor type, together with complement, before preparing the chimaeric TsF. The TsF was then tested for its activity. The results are shown as Expt. 3 of Table 2. The chimaeric TsF suppressed both C57BL/6 and C3H responses. However, TsF obtained from the chimaeric thymocytes pretreated with antibody against H-2 of donor type failed to suppress the responses, whereas both B6 and C3H responses were suppressed by TsF from chimaeric thymocytes pretreated with anti-host antibody and complement. Therefore, both donor- and host-restricted TsF were produced by the cells bearing H-2 of the donor type.

In the second experiment, KLH-TsF derived from the chimaeric mice (either C3H→C57BL/6 or C57BL/6→C3H) that received host APC was applied to a column of anti-I-J^b or anti-I-J^k. The effluent and the acid eluate were tested for their suppressor activity on the C57BL/6 or C3H response. Table 3 shows that KLH-TsF from the C3H→C57BL/6 chimaera that received C57BL/6 APC suppressed the responses of both C3H and C57BL/6. However, after adsorption to the anti-I-J^b column, the chimaeric TsF lost its suppressor effect on the H-2^b response. The H-2^b-restricted suppressor activity was recovered in the eluate from the column. The opposite results were

Table 2 Genetically restricted suppression by KLH-TsF obtained from chimaeric mice

Origin of KLH-TsF	Anti-DNP IgG PFC per 10 ⁶ cultured cells			DNP-OVA	
	DNP-KLH				
	C57BL/6	C3H	BALB/c	C57BL/6	C3H
Expt 1					
—	4,510 ± 370	2,210 ± 190	2,440 ± 240	840 ± 40	790 ± 80
C3H	3,910 ± 230	210 ± 170	2,250 ± 260	990 ± 200	750 ± 20
C3H → C57BL/6	4,690 ± 670	730 ± 240	2,290 ± 240	1,010 ± 110	830 ± 110
C3H → C57BL/6 + C57BL/6 APC	920 ± 200	610 ± 190	2,450 ± 370	910 ± 110	760 ± 70
C57BL/6	800 ± 210	2,430 ± 440	2,370 ± 290	1,010 ± 90	870 ± 100
C57BL/6 → C3H	880 ± 270	2,290 ± 360	2,050 ± 240	910 ± 170	820 ± 20
C57BL/6 → C3H + C3H APC	1,160 ± 400	590 ± 60	2,530 ± 290	970 ± 120	810 ± 110
BALB/c	3,990 ± 210	2,360 ± 250	440 ± 70	ND*	ND
Expt 2					
—	4,550 ± 510	1,430 ± 80	ND	700 ± 70	490 ± 100
B10.A(3R)	490 ± 130	1,510 ± 150	ND	720 ± 100	430 ± 80
B10.A(3R) → C57BL/6	1,090 ± 290	1,450 ± 250	ND	660 ± 100	470 ± 40
B10.A(3R) → C3H	1,340 ± 150	1,370 ± 250	ND	670 ± 40	430 ± 70
B10.A(5R)	4,160 ± 170	290 ± 80	ND	750 ± 50	450 ± 60
B10.A(5R) → C57BL/6	3,540 ± 480	500 ± 70	ND	670 ± 130	470 ± 40
B10.A(5R) → C3H	4,150 ± 270	500 ± 140	ND	660 ± 60	450 ± 120
Expt 3					
—	4,120 ± 450	1,970 ± 220	3,670 ± 430	1,080 ± 80	1,000 ± 80
C57BL/6	800 ± 180	1,960 ± 260	3,890 ± 60	1,050 ± 60	1,140 ± 100
C3H	4,050 ± 190	410 ± 50	4,120 ± 100	1,150 ± 60	1,090 ± 50
C57BL/6 → C3H + C3H APC	1,000 ± 140	280 ± 40	3,850 ± 110	1,120 ± 70	1,070 ± 180
C57BL/6 → C3H + C3H APC treated with C	4,030 ± 430	2,050 ± 160	3,930 ± 280	1,160 ± 40	1,010 ± 190
C57BL/6 → C3H + C3H APC treated with anti-H-2D ^b + C	1,010 ± 140	400 ± 80	3,830 ± 360	1,070 ± 190	990 ± 150
C57BL/6 → C3H + C3H APC treated with anti-H-2K ^k + C	3,880 ± 180	1,760 ± 250	360 ± 430	1,120 ± 80	1,000 ± 40
BALB/c					

KLH-specific TsF (KLH-TsF) was prepared by freezing and thawing of thymocytes from chimaeric or non-chimaeric mice that had been primed with 200 µg of KLH twice at a 2-week interval as described elsewhere³. In some experiments, the chimaeric mice were injected intravenously twice with 2 × 10⁶ APC (anti-Thy-1-treated peritoneal exudate cells) of the host type at the same time as immunization with KLH. Peritoneal exudate cells were prepared according to the method described elsewhere²³, and treated with anti-Thy-1 and complement (C). The phenotypes of peritoneal exudate cells used were determined in a cytotoxicity assay, and over 95% were found to be Ia-positive cells, with no T-cell contamination (no Thy-1⁺, Lyt-1⁺ or Lyt-2⁺). Two weeks after the second immunization with KLH, the thymocytes derived from chimaeric mice were used to prepare KLH-TsF. In experiment 3, KLH-primed thymocytes (1 × 10⁷ ml⁻¹) derived from chimaeras that received APC of the host type, were treated with anti-H-2D^b (anti-donor) or anti-H-2K^k (anti-host) antibody plus complement, washed, then subjected to freezing and thawing for TsF preparation. KLH-TsF (25 µl, a concentration equivalent to 1 × 10⁷ thymocytes ml⁻¹) was added at the start of cultivation to spleen cell cultures (1.6 × 10⁶ per well) of C57BL/6 or C3H mice primed 4 weeks previously with 100 µg DNP-KLH or 0.1 µg ml⁻¹ DNP-OVA plus 10⁹ *Bordetella pertussis* vaccine. The cells were cultured for 5 days in a 48-well flat-bottomed plate (Costar 3548) with 0.4 ml of RPMI-1640 medium enriched with 10% FCS in the presence of 0.01 µg ml⁻¹ DNP-KLH or 0.1 µg ml⁻¹ DNP-OVA. Anti-DNP IgG PFC were enumerated by using DNP-coupled sheep erythrocytes. Numbers are the mean (±s.d.) PFC number for three cultures. ND, not determined.

Table 3 Existence of two distinct TsF bearing I-J^b and I-J^k determinants in chimaeric TsF

Origin of KLH-TsF	Column	Materials added	Anti-DNP IgG PFC response in	
			C57BL/6	C3H
—	—	—	3,150 ± 190	1,330 ± 120
C3H → B6 + B6 APC	—	Unadsorbed	530 ± 240	190 ± 140
C3H → B6 + B6 APC	Anti-I-J ^b	Effluent	3,110 ± 150	170 ± 90
C3H → B6 + B6 APC	Anti-I-J ^b	Eluate	730 ± 400	1,330 ± 100
C3H → B6 + B6 APC	Anti-I-J ^k	Effluent	840 ± 340	1,370 ± 180
C3H → B6 + B6 APC	Anti-I-J ^k	Eluate	3,430 ± 400	230 ± 60
B6 → C3H + C3H APC	—	Unadsorbed	670 ± 200	360 ± 110
B6 → C3H + C3H APC	Anti-I-J ^b	Effluent	3,170 ± 300	390 ± 120
B6 → C3H + C3H APC	Anti-I-J ^b	Eluate	400 ± 80	1,470 ± 180
B6 → C3H + C3H APC	Anti-I-J ^k	Effluent	210 ± 200	1,440 ± 70
B6 → C3H + C3H APC	Anti-I-J ^k	Eluate	3,160 ± 390	470 ± 160

KLH-TsF (100 µl, equivalent to 1.5 × 10⁷ thymocytes) from chimaeric mice that received APC of the host type (same materials as used in Table 2) was applied to and incubated with immunoabsorbent columns of anti-I-J^b or anti-I-J^k antiserum for 1 h at 4 °C. The effluent or the acid eluate (0.175 M glycine-HCl buffer, pH 3.2) was dialysed, concentrated to a final volume of 100 µl with Sephadex powder, added to a culture of DNP-KLH-primed spleen cells of C57BL/6(B6)(H-2^b) and C3H(H-2^k) mice, and tested for its suppressor activity (see Table 2). Conventional anti-I-J antisera were used in this experiment to detect any types of I-J determinants generated in the chimaera. Values are mean number of PFC (±s.d.) for three cultures, expressed as number per 10⁶ cultured cells.

obtained when the same KLH-TsF was applied to an anti-I-J^k column. The H-2^k-restricted TsF was adsorbed to and eluted from the anti-I-J^k column, whereas the H-2^b-restricted TsF was recovered in the effluent. Similar results were obtained for KLH-TsF from the C57BL/6 → C3H chimaera that received C3H APC: the H-2^b-restricted KLH-TsF was recovered in the effluent while the H-2^k-restricted KLH-TsF was obtained in the eluate from the anti-I-J^k column. These results demonstrate that the chimaeric TsF contains two types of TsF bearing I-J of host or donor type, that is, I-J^b-TsF or I-J^k-TsF. Hence, both donor- and host-restricted Ts (anti-H-2^k and anti-H-2^b T cells), each of which produce I-J^k- or I-J^b-TsF, are generated from C3H(H-2^k) bone marrow cells under the influence of H-2^b. C57BL/6(H-2^b) bone marrow cells also produce I-J^k-TsF in the H-2^k environment, implying that I-J of the host type is generated in the chimaera. It is probable, therefore, that I-J represents the idiotype determinant on the variable part of the self-receptor of Ts, as speculated by Schrader¹⁹, and recently discussed by Kurata *et al.*²⁰ and Murphy²¹, and also that expression of the I-J phenotype on KLH-TsF is dictated by the host environment.

If the I-J is the idiotype determinant of the recognition component on TsF, several questions must be answered. First, why does the I-J on TsF or Ts receptor map in the H-2 complex? There is no definite answer to this question as yet. However, the experiments using chimaeras described in Table 2 suggest the existence of a new H-2-encoded class II-like molecule on APC. This molecule seems to be essential for the generation and clonal expansion of I-J⁺ Ts and also necessary for expression of the I-J phenotype. The molecule is apparently expressed on APC at least (Tables 2, 3), but does not seem to be identical to the known Ia molecule (that is I-A or I-E). In fact, the B10.A(5R) → C57BL/6 chimaera produces only the H-2^k-restricted TsF(I-J^k-TsF) even though B10.A(5R) and C57BL/6 mice have the I-A^b subregion in common, and I-A^b APC should be present in the chimaera (Table 2). Involvement of the I-E molecule is also excluded, because the H-2^b mice do express the I-J^b phenotype, although they are unable to express the I-E molecule²².

Second, why do Ts and TsF express I-J with H-2-haplotype specificity? If the I-J idio type is expressed on the self-recognizing structure (the I-J⁺ molecule), the I-J could be complementary to the MHC-encoded molecule on the APC so that the I-J seems to be a reflection of MHC-encoded molecule. If this is the case, the variable (V) gene repertoire of the self-receptor of Ts should be limited. In any event, the genetic identification and biochemical characterization of the molecules on APC will be important for resolving the I-J paradox.

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I-J epitopes are adaptively acquired by T cells differentiated in the chimaeric condition

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I-J has been defined as a locus mapped in the murine major histocompatibility complex (MHC) which encodes serological markers found primarily on the surface of suppressor T cells (Ts) and soluble suppressor factors (TsF)^{1,2}. Recent studies have, however, revealed that there is no such specialized locus within the MHC at the DNA level^{3,4}. As the existence of I-J determinants at the protein level on functional T cells, T-cell clones and hybridomas has been confirmed by several serological and biochemical studies⁵⁻⁷, this contradiction has raised serious arguments in the immunological community concerning the nature, origin and expression of I-J determinants. We have raised a number of monoclonal antibodies against the polymorphic structure of I-J molecules, and have studied the expression of I-J epitopes on T cells derived from irradiated bone marrow chimaeras in which stem cells of different genotype differentiated into T cells under the foreign host MHC environment. The results, presented here, indicate that I-J epitopes are not primarily determined by the MHC genes of the stem cells themselves, but are adaptively acquired by T cells differentiated in the chimaeric condition according to the environmental MHC phenotype. Thus, the serologically detectable I-J epitopes are found to be associated with inducible T-cell receptors recognizing self class II MHC antigens.

We have previously reported an interesting activity of anti-I-J monoclonal antibody, which was used to determine serologically the allelic forms of I-J molecules on T cells⁸. The essential findings were: (1) anti-I-J^k antibody blocks the syngeneic and allogeneic mixed lymphocyte reactions (MLRs) of H-2^k and H-2^a haplotype strains but not that of their H-2 congenic partners (this was caused by the blocking of responding T cells but not of stimulator cells). (2) In the syngeneic MLR of H-2^{k/b} F₁ T cells stimulated by F₁ or either parental strain, anti-I-J^k antibody inhibited the response to H-2^k but not to H-2^b stimulators, indicating that I-J^k epitopes are expressed only on those F₁ responder T cells recognizing H-2^k stimulator molecules but not on those responding to H-2^b stimulators. (3) The pattern of inhibition of the syngeneic and allogeneic MLRs of T cells from individual H-2^k strains by various anti-I-J^k antibodies was different under the control of as yet undetermined non-MHC background genes. Thus, the I-J epitopes were found to be associated with the MLR recognition sites of T cells, which are not determined solely by the H-2 genes but which are also influenced by unknown non-MHC genes. These results prompted us to examine the expression of I-J on T cells of irrelevant H-2 genotypic origin but differentiated in the chimaeric condition.

To determine the nature of I-J epitopes on chimaeric T cells, we also used another set of monoclonal antibodies reactive with I-region-controlled epitopes uniquely expressed on T but not on B cells⁹. These monoclonal antibodies were produced by immunizing A.TH (H-2^d) mice with I-region-incompatible A.TL (H-2^l) lymphoid cells; they were selected for their specific reactivity with some functional T cells and T-cell hybridomas⁹. The antibodies were unable to react with conventional class II antigens on B cells and macrophages, but were able to precipitate a unique polypeptide of relative molecular mass 33,000 (33 K), which is apparently under the control of the I-A subregion of the H-2 complex, from a T-cell hybridoma producing an antigen-specific augmenting T-cell factor (TaF)¹⁰. The interrelationship between this polypeptide and the α and

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Table 1 Inhibition of allogeneic MLR of normal and chimaeric mice by anti-I-J and anti-Iat monoclonal antibodies

Responder Antibody	C3H Δc.p.m. % Inh.	B6 Δc.p.m. % Inh.	B6C3F ₁ Δc.p.m. % Inh.	B6C3F ₁ → C3H Δc.p.m. % Inh.	B6C3F ₁ → B6 Δc.p.m. % Inh.	C3H → B6C3F ₁ Δc.p.m. % Inh.	B6 → B6C3F ₁ Δc.p.m. % Inh.	B6 → C3H Δc.p.m. % Inh.	C3H → B6 Δc.p.m. % Inh.
Complement alone	100,858 0	91,457 0	101,450 0	31,367 0	62,938 0	50,095 0	38,234 0	34,699 0	117,835 0
Anti-I-J 1G8	44,620 52	90,296 1	59,101 42	6,976 78	59,155 6	3,786 93	33,417 13	36,035 -4	121,349 -3
4B11	113,105 -2	100,420 -9	104,912 -3	31,618 0	61,910 1	55,157 -10	39,890 -4	32,222 7	116,697 1
KN34	43,608 61	85,610 6	60,377 40	11,372 64	59,953 5	22,344 59	11,419 70	14,758 57	115,532 2
Anti-Iat IL9	56,979 49	87,721 4	60,045 41	6,260 80	63,786 -1	27,518 50	35,891 6	34,148 2	123,705 -5
2L2	62,001 44	83,034 9	59,422 41	9,007 71	63,260 0	24,287 55	17,511 54	13,315 62	117,154 1
14P	106,987 3	93,849 -3	96,793 4	29,875 5	59,669 5	53,494 3	40,657 -6	33,671 0	125,444 -6
74L	103,709 6	91,894 -1	94,503 7	30,666 2	59,669 5	57,069 -3	37,620 2	33,949 2	115,352 2

Inhibition of MLR by the cytotoxic treatment of responder cells from normal and chimaeric mice with anti-I-J and anti-Iat antibodies. An allogeneic MLR (allo-MLR) was set up with 2.5×10^5 spleen cells as responders and an equal number of MMC-treated SJA/9 spleen cells as stimulators in RPMI 1640 medium supplemented with 10% fetal calf serum and 5×10^{-5} M 2-mercaptoethanol in 96-well flat-bottomed microtitre plates. Cells were cultured for 5 days, and DNA synthesis assessed by pulsing with $1 \mu\text{Ci}$ of ^3H -thymidine for the final 16 h. The inhibitory activity of each antibody was studied by treating responder cells for 45 min at room temperature with a 1:10 dilution of ascites fluid from BALB/c *nu/nu* mice containing the monoclonal antibody followed by incubation with a 1:10 dilution of selected rabbit complement (C) for 30 min at 37 °C. After extensive washing, cells were cultured with MMC-treated stimulator cells. The mean c.p.m. for five replicate cultures was determined, and the values shown are net c.p.m. (Δc.p.m.), calculated by subtracting the sum of the c.p.m. for stimulator and responder cultures from the c.p.m. of a mixture of the two types of cells. Per cent MLR inhibition (% Inh.) was calculated as: $[1 - (\Delta\text{c.p.m. for MLR with antibody} + \text{C} / \Delta\text{c.p.m. for MLR with C alone})] \times 100$. Note that the response of C3H and B6C3F₁, but not B6, was inhibited by treatment with two of the anti-I-J antibodies (1G8 and KN34) and with two of the anti-Iat antibodies (IL9 and 2L2). The MLR of the B6C3F₁ → C3H and C3H → B6C3F₁ chimaeras was inhibited by the same four antibodies that inhibited the parental C3H response, whereas no inhibition of the MLR was observed with spleen cells from the B6C3F₁ → B6 chimaera. The MLR of the B6 → B6C3F₁ chimaera and of the fully allogeneic B6 → C3H chimaera stimulated with SJA/9 was inhibited by only two of the antibodies (2L2 and KN34). Similar, even more prominent blocking of the MLR, with the same inhibitory pattern, was observed by the simple addition of $1 \mu\text{g ml}^{-1}$ of each antibody at the start of cultivation (data not shown).

β chains of T-cell receptors is unknown. Because the epitopes detected by these antibodies are selectively expressed on T cells, and as I region apparently controls the molecule, we designated these monoclonal reagents as anti-Iat antibodies. The present experiments used anti-I-J and anti-Iat antibodies, the properties of which have been described fully elsewhere⁸⁻¹¹.

Fully allogeneic and semi-allogeneic bone marrow chimaeras were produced according to the protocol of Singer *et al.*¹². The mice were maintained for over 3 months to establish a complete chimaeric state, which was confirmed at the time of each experiment by cytotoxicity assays using appropriate anti-H-2 reagents and by the unresponsiveness to both host and donor type stimulator cells in MLR. Sera from individual chimaeric mice were also typed to confirm the immunoglobulin allotype of donor origin.

The allo-MLR was performed by culturing 2.5×10^5 spleen cells from normal or chimaeric animals with an equal number of mitomycin C (MMC)-treated spleen cells of strain SJA/9 (*H-2^s*, *Igh-1^a*) in 0.2 ml for 5 days in 96-well microtitre plates. Although any MHC-incompatible strains can be used as stimulators, SJA/9 was selected for use because both C3H and C57BL/6 (B6) responded very well to the SJA/9 cells, and the response of C3H was found to be inhibitable by a panel of anti-I-J and anti-Iat antibodies. Three anti-I-J (1G8, IgM; 4B11, IgM; and KN34, IgG2b) and four anti-Iat (IL9, IgM; 2L2, IgG2b; 14P, IgG1; and 74L, IgG2a) monoclonal antibodies were used in the present experiment to identify the epitopes on responding T cells. Before MLR cultivation, responder cells were treated for 45 min at room temperature with a 1:10 dilution of ascites fluid from BALB/c *nu/nu* mice containing each monoclonal antibody, then incubated at 37 °C for 30 min with a 1:10 dilution of selected rabbit complement. For each antibody, less than 10% of responder cells were killed by the treatment. In the blocking experiments, the globulin fraction of ascites fluid was added to the MLR at a concentration of $1 \mu\text{g ml}^{-1}$ at the start of cultivation. Cells were pulsed with $1 \mu\text{Ci}$ of ^3H -thymidine (^3H -TdR) for the final 16 h of the 5-day cultivation.

Table 1 shows the inhibition of uptake of ^3H -TdR by C3H and (B6 × C3H)F₁ (B6C3F₁) responder cells stimulated with MMC-treated SJA/9 spleen cells by the treatment of the responder cells with two anti-I-J (1G8 and KN34) and two anti-Iat (IL9 and 2L2) antibodies. The response of B6 cells was not inhibitable by treatment with any of these antibodies. The MLR of spleen cells derived from the B6C3F₁ → C3H chimaera was also inhibitable by all four antibodies that were effective in inhibiting the response of parental C3H. None of the mono-

clonal antibodies was effective in inhibiting the response of spleen cells from the B6C3F₁ → B6 chimaera, even though the responding cells carried an *H-2^k* determinant, as detected by cytotoxicity testing with appropriate anti-class I antibodies. Thus, the T cells of F₁ stem-cell origin were found to express I-J^k and Iat^k epitopes only when they differentiated in the environment of an *H-2^k* but not an *H-2^b* recipient.

The experiments using parental T cells transferred into F₁ chimaeras gave more definitive results. Cells responding to SJA/9 derived from C3H → B6C3F₁ chimaeras were killed by all four antibodies (1G8, KN34, IL9 and 2L2), while those from B6 → B6C3F₁ chimaeras expressed at least two of the epitopes detected by anti-I-J^k (KN34) and anti-Iat^k (2L2). The latter finding was especially interesting, as it indicates that the T cells of *H-2^b* stem-cell origin which differentiated in the *H-2^{k × b}* environment acquired at least some of the I-J^k and Iat^k phenotypes. The parental haplotype origin of the responding T cells was always confirmed by cytotoxicity testing with appropriate anti-H-2 reagents. In the fully allogeneic chimaera, the response of the B6 → C3H chimaera was inhibitable by the antibodies 2L2 and KN34, whereas the response of the C3H → B6 chimaera was not inhibitable by any of the antibodies. The same pattern of blocking of allo-MLR was observed by adding $1 \mu\text{g ml}^{-1}$ of these antibodies to the MLR culture (data not shown), which suggested the direct association of I-J and Iat epitopes with the allorecognition site of MLR responding T cells. The results indicate that I-J^k and Iat^k epitopes on MLR responder T cells are adaptively acquired in the chimaera, where allogeneic or semi-allogeneic stem cells differentiate into mature T cells under the selective influence of environmental MHC products.

Note, however, that the responses of F₁ → C3H and C3H → F₁ chimaeras were inhibited by the same four antibodies that were effective in inhibiting the MLR of normal C3H cells responding to the same SJA/9 stimulator, whereas the responses of B6 → F₁ and B6 → C3H chimaeras to the same stimulator were inhibited by only two of the four antibodies. This result suggests that the expression of I-J and Iat epitopes on the alloreactive T cells is determined essentially by two different factors: (1) the environmental MHC products in the host under which stem cells differentiate into T cells and acquire the alloreactive repertoire, probably in the thymus; (2) possibly the non-*H-2* genomic material originally possessed by the stem cell itself. Thus, B6 stem cells differentiated into T cells in a C3H environment are unable to express all the epitopes possessed by normal C3H responder cells.

We tested the above postulate by examining the pattern of inhibition of the MLR of bone marrow chimaeras transplanted

with *H-2*-compatible stem cells having different non-*H-2* background genes. We have shown previously that the inhibition pattern of both the syngeneic and allogeneic MLR by several anti-I-J antibodies differs among *H-2^k* strains, under the control of as yet unknown non-MHC genes⁸. Therefore, we constructed bone marrow chimaeras of B10.BR→C3H, in which *H-2* is identical, whereas non-*H-2* genes are different. Table 2 compares the inhibitory patterns produced by treatment with several anti-I-J and anti-Iat antibodies of B10.BR, C3H and B10.BR→C3H chimaeric T cells responding to the same SJA/9 stimulator. Whereas the C3H response was inhibited by 1G8, KN34, 1L9 and 2L2, the response of B10.BR was inhibited by only 1G8 and 74L, reflecting the difference in non-MHC genes between these two *H-2^k* strains. Exactly the same inhibitory patterns were observed in the blocking experiment in which antibodies were simply added to the MLR culture. The chimaeric T cells of B10.BR genotype differentiated in the C3H environment showed exactly the same pattern as B10.BR cells, but not the same as C3H, indicating that the expression of I-J and Iat epitopes is genetically determined in the case of *H-2*-compatible chimaeras.

These results are of importance in understanding why I-J products are detectable by serological and biochemical means^{5-7,11,13-16}, yet no genes have been identified at the prescribed position in the MHC^{3,4}. Several recent reports have discussed possible explanations for this discrepancy: (1) the I-J molecule is a modified α or β polypeptide of a class II MHC antigen, created by post-translational modification¹⁷; (2) I-J epitopes are created via an interaction between MHC and non-MHC genes, the latter being designated as Jt and mapped to chromosome 4²³; and (3) I-J is not the direct product of an MHC gene but is an inducible T-cell receptor for self I-region products^{11,18}.

Our findings indicate the following: (1) I-J and related Iat epitopes are present at the allorecognition site of MLR responder T cells; (2) I-J^k and Iat^k epitopes are inducible on chimaeric T cells derived from strains with a different MHC genotype but differentiated in the *H-2^k* environment; (3) the expression of I-J^k and Iat^k epitopes in chimaeric conditions is determined not only by the *H-2* phenotype of the host but also by the genomic material originally possessed by the stem cells; (4) the distribution patterns of I-J and Iat epitopes are determined by as yet unknown non-*H-2* genes. Based on these findings, we believe that I-J and Iat epitopes are not the mere markers of functional T-cell subsets, as originally proposed, but are associated with inducible T-cell receptors for alloantigen whose repertoire is determined by the environmental MHC products encountered during an early stage of T-cell differentiation. It has been proposed that such allorecognition is a version of self-MHC-restriction which is determined by selection in the thymus¹⁹⁻²².

Hayes *et al.*^{23,28} have suggested that the MHC and the *Jt* locus on chromosome 4 interact to give expression of the I-J epitope, probably through glycosylation. It is, however, difficult to explain the adaptive acquisition of polymorphic epitopes by means of simple glycosylation, because the *F₁*→B6 and C3H→B6 chimaeras cannot express I-J^k even though the stem cells should carry both *H-2* and *Jt* genes. It is puzzling that certain I-J epitopes are not detected on B10.BR responding T cells regardless of their presence on B6→C3H chimaeric T cells. Obviously there exists an as yet unknown regulatory mechanism whereby the fine organization of I-J epitopes at T-cell receptor sites is determined. Our results do not favour the postulate that I-J is a modified α or β chain of a class II antigen¹⁷, as it is unlikely that such pleomorphic molecules can make structural changes by adapting to the environmental class II antigens.

It has been well documented that the functional MHC-restriction specificities of T cells are adaptable, changing according to the *H-2* environment in which they undergo early differentiation in the thymus^{21,22,24-26}. Yamauchi *et al.*²⁷ demonstrated adaptive changes in the functional MHC and immunoglobulin heavy-chain variable-region restriction specificities of sup-

Table 2 Inhibition of the allo-MLR of T cells from the B10.BR→C3H chimaera by anti-I-J and anti-Iat monoclonal antibodies in comparison with B10.BR and C3H T cells

Responder cells:		B10.BR		C3H		B10.BR → C3H	
Monoclonal antibody		Δc.p.m.	% Inh.	Δc.p.m.	% Inh.	Δc.p.m.	% Inh.
C alone		59,887	0	48,992	0	36,775	0
Anti-I-J	1G8	26,545	56	19,151	61	15,368	58
	4B11	59,393	1	45,578	5	35,728	3
	KN34	58,247	3	18,489	62	36,862	0
Anti-Iat	1L9	61,245	-2	22,691	54	36,655	0
	2L2	58,389	3	27,007	45	34,364	7
	14P	57,059	5	54,272	6	34,328	7
	74L	33,806	44	42,242	13	18,339	50

Inhibition of the MLR by treatment of responder cells from normal and *H-2*-compatible chimaeric mice with anti-I-J and anti-Iat antibodies. Allo-MLRs of B10.BR, C3H and B10.BR→C3H chimaeras stimulated by MMC-treated SJA/9 spleen cells were set up as described for Table 1. Note that the MLR of B10.BR is inhibited by two antibodies (1G8 and 74L), one of which (74L) does not inhibit the MLR of C3H. The inhibitory pattern of the B10.BR→C3H chimaera is exactly the same as that of B10.BR but differs from that of C3H.

pressor factors derived from chimaeric T cells, but were unable to detect the adaptive acquisition of allogeneic I-J determinants. Here, by using monoclonal antibodies that detect the polymorphic portion of the I-J molecule, we have provided evidence that serologically detectable I-J and Iat epitopes associated with the allorecognition structure are adaptively acquired in the chimaeric condition. We have shown previously that different anti-I-J monoclonal antibodies killed T cells responding to different alloantigens, which indicates the clonal distribution of these epitopes⁸. Thus, we conclude that I-J and Iat epitopes are not directly encoded by intra-*H-2* genes but are associated with the T cells' inducible receptors for the self class II antigens that determine the restriction specificity. This explains the apparent linkage of I-J and Iat phenotypes to class II alleles. Although I-J has yet to be mapped within the MHC, the present results provide new insight into the T-cell receptor for self antigens, and anti-Iat reagents should be useful in defining the restriction specificities and development of T cells.

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T cells with receptors for IgD

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The role of IgD in the immune response has been elusive, although its predominance on the cell surface suggests a receptor function. We have shown previously that euthymic but not athymic BALB/c mice, injected with IgD before antigen, exhibit enhanced antibody responses which can be transferred by T cells^{1,2}. Isotype-specific T cells have been reported to have both upward and downward immunoregulatory effects³⁻⁵. Here we demonstrate the existence of T cells with receptors for IgD, and show that exposure to IgD *in vivo* or *in vitro* significantly increases the number of T δ cells in the spleen and lymph nodes but not in the thymus. The kinetics of T δ -cell appearance *in vivo* parallels that of the immunoenhancing effect which occurs after injection of IgD. These T δ cells are of the Lyt 1⁺2⁻ T-cell phenotype.

Recently, we have found that mice bearing IgD-secreting plasmacytomas have enhanced humoral immune responses¹. We postulated that T cells bearing receptors for IgD (T δ) mediate the increased antibody production. The potential immunoregulatory activity of such a T-cell population is underlined by the fact that most peripheral B cells carry membrane IgD⁶.

We used sheep erythrocytes (SE) coupled to IgD in a rosetting assay to enumerate IgD-specific rosette-forming cells (IgD-RFC). The effects of overnight incubation at 37 °C of normal spleen cells with either a 1:10 dilution of TEPC 1017 ascites fluid (containing ~3 mg IgD ml⁻¹) or 0.04–20 µg of purified IgD ml⁻¹ are shown in Table 1 and Fig. 1. Fresh normal BALB/c spleen cells show 5 ± 0.8% IgD-RFC (*n* = 15) (result not shown), and after incubation with medium this percentage remains essentially unchanged. In contrast, exposure to IgD for

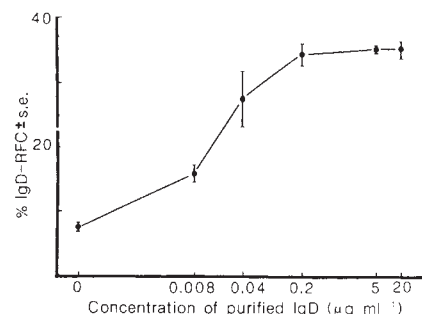


Fig. 1 Dose-response of IgD-induced IgD-RFC. Spleen cells from BALB/c mice were incubated at 37 °C for 18 h in 1 ml of MEM containing 2% FCS without or with purified IgD as described in Table 1. Following this incubation period, cells were washed twice in MEM and then examined in the IgD-RFC assay as described in Table 1.

18 h causes the percentage RFC to increase to >25%. Similarly, purified splenic T cells incubated with IgD yield >25% RFC (Table 1). The absence of an increase in percentage RFC on purification of T cells after panning on anti-immunoglobulin-coated dishes could be the result of selective adherence of a proportion of T δ cells on the membranes of IgD-expressing B cells. Overnight incubation of splenic T cells with IgM- and IgG-containing ascites does not cause an increase in IgD-RFC. Addition of IgD to the RFC assay mixture significantly reduces the number of IgD-RFC detected (Table 1), whereas addition of IgM- and IgG-containing ascites has no detectable inhibitory effect.

Using a similar *in vitro* approach, we examined the effect of exposure to IgD on various cell populations (Table 2). Whereas spleen cells from normal BALB/c mice show a 5–6-fold increase in the number of IgD-RFC to 28% after *in vitro* incubation with IgD, spleen cells from mice injected with IgD *in vivo* show a mean value of 30% IgD-RFC with or without additional *in vitro* exposure to IgD. The failure of these cells to respond with a further increase in the percentage of IgD-RFC suggests that this 30% value represents an upper limit to the inducible level of such cells.

Table 1 Specificity of IgD-RFC induced by exposure to IgD *in vitro*

Cell source	Addition during RFC assay*		Mean % IgD-RFC ± s.e. (<i>n</i>) after overnight incubation with:			
	IgD	IgM + IgG	Medium alone	IgD-containing ascites fluid	Purified IgD	IgM + IgG-containing ascites fluid
Spleen cells	—	—	8 ± 0.7 (9)†‡	27 ± 1.6 (10)†	29 ± 1.9 (19)‡	6 ± 1.3 (3)
Splenic T cells	—	—	9 ± 0.8 (4)§	31 ± 3.0 (4)§	27 ± 1.2 (8)	7 ± 0.9 (6)
Splenic T cells	+	—	—	6 ± 2.5 (3)¶	—	—
Splenic T cells	—	+	—	30 ± 0.05 (3)	—	—

Cells were obtained from BALB/c mice. Splenic T cells were purified by negative selection by panning spleen cells on Petri dishes coated with affinity-purified goat anti-mouse immunoglobulin¹⁴. The percentage of immunoglobulin-positive cells contaminating such purified T-cell suspensions following this T-cell enrichment procedure was consistently <1%. Cells (2.5×10^6) were incubated at 37 °C for 18 h in 1 ml of minimal essential medium (MEM, Gibco) containing 2% fetal calf serum (FCS). Where indicated, the medium was supplemented with TEPC 1017 ascites fluid to 3 mg IgD ml⁻¹, 0.04–20 µg ml⁻¹ purified IgD (pooled data from Fig. 1), or 10% IgM + IgG-containing ascites fluid obtained from mice bearing the MOPC 104E plasmacytoma. Following this incubation period, cells were washed twice and the cell concentration readjusted to 2.5×10^6 ml⁻¹ in MEM + 2% FCS. The cells were then used in the RFC assay. IgD was affinity-purified from ascites fluid taken from BALB/c mice bearing the IgD-secreting myeloma TEPC 1017, according to methods described previously¹, then it was coupled to SE using the CrCl₃ coupling method¹⁵. Coupling of IgD to SE was confirmed by both positive haemagglutination and indirect immunofluorescence assays using a rabbit anti-mouse IgD antiserum. IgD-RFC were enumerated using a modified method of Chen *et al.*¹⁶. Briefly, 0.2 ml of 1% IgD-SE was mixed with 0.1 ml of assay cells (2.5×10^5) at 37 °C for 15 min. Cells were then centrifuged at 500 r.p.m. (200g) for 5 min and incubated further at 4 °C for 45 min or overnight. Immediately before scoring RFC, the lymphocytes were stained using 0.025 ml of a 1% toluidine blue solution. Lymphocytes surrounded by over three indicator cells were scored as rosettes and the result expressed as % RFC. In each experiment, the percentage of cells forming rosettes with SE was determined and always found to be <2%.

* Where indicated, IgD-containing, or IgM plus IgG-containing ascites fluid was added to samples to a final concentration of 6%.

† For medium alone compared with IgD-containing ascites fluid, *P* < 0.0001.

‡ *P* < 0.0001; § *P* = 0.001; || *P* < 0.001; ¶ *P* = 0.001.

Table 2 *In vitro* induction of T δ cells following overnight incubation with IgD-containing ascites fluid

Cell source	<i>In vivo</i> pretreatment	Mean % IgD-RFC $\pm$ s.e. following overnight incubation with:		<i>P</i>
		Medium alone	IgD-containing ascites fluid	
Spleen cells	—	8 $\pm$ 1.2	28 $\pm$ 1.9	<0.0001
Spleen cells	TNP-KLH*	11 $\pm$ 1.7	30 $\pm$ 2.4	0.001
Spleen cells	IgD†	30 $\pm$ 1.7	31 $\pm$ 2.4	NS
Splenic T cells	—	8 $\pm$ 0.7	30 $\pm$ 2.9	0.01
Splenic Lyt 1 ⁺ 2 ⁻ T cells	—	—	40 $\pm$ 2.1	<0.0001‡
Splenic L3T4 ⁻ T cells	—	—	8 $\pm$ 1.9	NS‡
Splenic B cells	—	5 $\pm$ 0.7	8 $\pm$ 1.4	NS
Lymph node cells	—	5 $\pm$ 0.9	42 $\pm$ 3.9	0.006
Thymocytes	—	5 $\pm$ 0.3	8 $\pm$ 1.5	NS
C.B20 spleen cells	—	8 $\pm$ 0.5	31 $\pm$ 2.2	<0.0001

Cells were incubated in MEM + 2% FCS with or without IgD-containing ascites fluid as described in Table 1. The results are the means for 3–6 assays. Except for C.B20 spleen cells, the cells were obtained from BALB/c mice. Splenic T cells were purified as described in Table 1. Using purified T-cell suspensions as the starting population, Lyt 1⁺2⁻ and L3T4⁻ T-cell subpopulations were isolated by complement-mediated cytotoxicity using the monoclonal antibodies anti-Lyt 2.2 (19/178), provided by Dr U. Hammerling (Sloan-Kettering Institute, New York), and anti-L3T4 (GK1.5)¹⁷, obtained from Dr F. Fitch (University of Chicago, Illinois) respectively. Splenic B cells were purified by complement-mediated cytotoxicity of T cells using a cocktail containing anti-Thy-1.2 (6.80), provided by Dr U. Hammerling, anti-Lyt 1.2 (C3PO) provided by Dr J. Klein (Max Planck Institute, Tübingen) and anti-L3T4 (GK1.5).

* Mice were injected intravenously (i.v.) with 100 μ g TNP-KLH 12 and 5 days before the day of assay.

† Mice were injected i.p. with 0.5 ml of IgD-containing ascites fluid 7 and 1 day before the day of assay.

‡ *P* values determined by comparing the mean % IgD-RFC following overnight incubation with IgD with the mean % RFC for splenic T cells following overnight incubation in medium alone.

The T-cell lineage of the IgD-RFC was investigated further by examining the ability of specific T-cell subsets to form rosettes with IgD-SE. As shown in Table 2, isolated Lyt 1⁺2⁻ but not L3T4⁻ T cells yield significantly increased numbers of IgD-RFC after overnight incubation with IgD. These results suggest that most, if not all, the IgD-RFC are Lyt 1⁺2⁻, L3T4⁺ T cells—that is, they have the helper cell phenotype. However, the possibility that some RFC may be Lyt 2⁺ cannot be excluded; further studies are needed to determine whether the incidence of IgD-RFC in Lyt 2⁺ cells can be increased if they are exposed to IgD in the presence of other cell types. Marcelletti and Katz⁷ have recently reported that optimal induction of IgE-binding, Lyt 2⁺ T ϵ cells is dependent on the presence of B cells. The majority of T α and T ϵ cells induced by exposure to IgA and IgE respectively, are Lyt 2⁺ (refs 8, 9).

The percentage of IgD-RFC in lymph-node cell suspensions also increases (from 5 to 42%) after incubation with IgD. In contrast to peripheral T cells, however, thymocytes show only background levels (5–8%) of IgD-RFC before and after *in vitro* exposure to IgD. Similarly, when isolated splenic B cells are incubated overnight with IgD, no significant increase above background numbers of IgD-RFC is seen.

The induction of IgD-RFC is not allotype-specific as spleen cells from C.B20 mice, which express the *b* allotype of IgD, are as responsive as BALB/c spleen cells to TEPC 1017-derived IgD of allotype *a*. In addition, C.B20 mice bearing the TEPC 1017 plasmacytoma show a significant enhancement of the 6-day primary response to trinitrophenyl-keyhole limpet haemocyanin (TNP-KLH) (523 $\pm$ 134% of the control for 19S plaque-forming cells (PFC) and 341 $\pm$ 141% of control for 7S PFC).

Although these data established that exposure to IgD *in vivo* and *in vitro* causes the appearance of IgD-RFC, the phenotype of the *in vivo*-induced RFC remained to be determined. Table

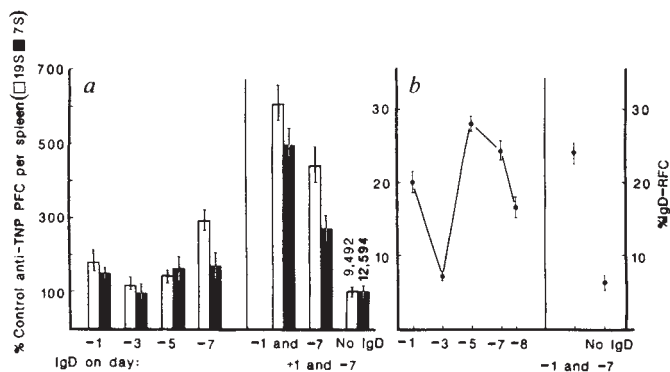


Fig. 2 *a*, Kinetics of IgD-induced enhancement of antibody response to TNP-KLH. Results are presented as % of control responses ($\pm$ s.e.). The geometric means of responses in control mice for 19S and 7S PFC per spleen (100%) are shown above the bars. BALB/c mice were injected with 0.5 ml of IgD-containing ascites fluid on the days indicated ($n=3-11$). Mice were primed on day 0 with 100 μ g of TNP-KLH and their anti-TNP PFC responses were measured 5 days later. Anti-TNP splenic PFC were assayed by the slide modification of the technique of Jerne *et al.*¹⁸. TNP-SE were prepared using the method described by Rittenberg and Pratt¹⁹. IgG-producing cells (that is, 7S responses) were developed with rabbit anti-mouse immunoglobulin in the complement and goat anti- μ in the agar. *b*, Kinetics of appearance of T δ cells after i.p. injection of IgD-containing ascites fluid. BALB/c mice were injected with 0.5 ml of IgD-containing ascites fluid on the days indicated. Spleen cells were obtained on day 0 and the percentage of IgD-RFC determined ($n=3-6$).

3 shows that ~30% IgD-RFC are obtained in both unfractionated and Lyt 1⁺2⁻ splenic T cells following injections of IgD. The induction of IgD-RFC *in vivo* also parallels the *in vitro* results in that >30% RFC are found in lymph nodes and only 3% RFC in thymus. The results in Tables 2 and 3 suggest strongly that injection of antigen also causes a significant increase in the number of splenic IgD-RFC. Further characterization of these RFC is underway.

The kinetics of appearance of T δ cells after intraperitoneal (i.p.) injection of IgD was studied in parallel with the humoral immune responsiveness of similarly treated mice (Fig. 2). The responses of animals injected with IgD on day -1, -5 or -7 or on both days +1 or -7 were higher than those of untreated mice. In contrast, the responses of mice injected with IgD 3 days before injection of antigen were not augmented. The presence or absence of augmentation of the PFC response is in complete agreement with the presence or absence of increased numbers of T δ cells in the spleen. However, the magnitude and kinetics of the two phenomena differ somewhat. The percentage of T δ cells rises to a near maximum within 1 day after IgD injection, falls to background levels by day 3 and then rises again to maximal levels by day 5. The percentage of T δ cells found in the spleens 1 day after a second injection of IgD is as high as after the first injection. However, the magnitude of the PFC response induced after two injections of IgD is much greater than at any time after a single injection. These findings suggest strongly that T δ cells are at least as readily induced as T cells bearing Fc receptors for other immunoglobulin isotypes^{8,10}, and that T δ cells have an augmenting effect on the 19S and 7S antibody responses. The observation that maximal augmentation of immune responses *in vivo* requires two injections, whereas maximal numbers of T δ cells are observed after a single injection of IgD, may reflect a ceiling effect with respect to the percentage of splenic T cells capable of expressing receptors for IgD. It is also possible that individual T δ cells either express more receptors for IgD or become more responsive to target B cells after repeated exposure to IgD.

Table 3 Phenotype and tissue distribution of T δ cells induced *in vivo*

Cell source	<i>In vivo</i> pretreatment with IgD- containing ascites fluid	Mean % IgD-RFC $\pm$ s.e. (n)	P
Spleen cells	+	23 $\pm$ 1.4 (8)	<0.0001
	—	5 $\pm$ 0.8 (6)	
Splenic T cells	+	30 $\pm$ 4.7 (5)	0.02
	—	4 $\pm$ 0.4 (4)	
Lyt 1 ⁺ 2 ⁻ splenic T cells	+	27 $\pm$ 1.3 (5)	<0.0005
	—	5 $\pm$ 0.3 (3)	
Lymph node cells	+	32 $\pm$ 2.5 (5)	<0.005
	—	12 $\pm$ 1.3 (4)	
Thymocytes	+	3 $\pm$ 0.8 (4)	NS
	—	5 $\pm$ 0.5 (4)	
TNP-KLH-primed spleen cells	—	12 $\pm$ 1.2 (7)	<0.005
Unprimed spleen cells	—	5 $\pm$ 0.6 (7)	

Cells were obtained from BALB/c mice. Splenic T cells and Lyt 1⁺2⁻ splenic T cells were purified as described in Tables 1 and 2. Where indicated, spleen cells were obtained from BALB/c mice immunized with 100 μ g TNP-KLH 5 days before the day of assay. Where indicated, mice were injected twice (i.p.) with 0.5 ml of IgD-containing ascites fluid 7 and 1 days before the day of assay.

Results from other studies show that augmentation of the humoral immune response in normal mice can be achieved by transfer of unfractionated spleen cells, unfractionated T cells² or purified Lyt 1⁺2⁻ T cells from donors preinjected with IgD¹¹. The mechanism of action of these IgD-induced T-helper cells has not yet been established, but, in the absence of polyclonal B-cell activation¹, it is expected that interaction of antigen with IgD on the surface of the B cell activates T δ cells, which then exert their augmenting effect. Whether, as suggested previously¹, this effect is mediated through down-regulation of IgD synthesis, a known consequence of B-cell activation by antigen or mitogen^{12,13}, or through the release of a soluble mediator, remains to be established. It is of interest to speculate that the small but significant increase in T δ cell number which occurs after injection of antigen is the result of stimulation by antigen-IgD complexes. Our demonstration of IgD-rosetting T cells provides evidence for the existence of a mechanism in which T-cell help is given to all IgD-positive B cells in an antigen-dependent though not antigen-specific fashion.

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Platelet secretory products inhibit lipoprotein metabolism in macrophages

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Macrophages possess a receptor that binds low-density lipoproteins (LDL) containing lysine residues modified by acetylation (Ac LDL), acetoacetylation (AcAc LDL) or malondialdehyde treatment^{1–3}. This receptor (referred to as the Ac LDL receptor or scavenger receptor) internalizes the bound lipoprotein. As a consequence, massive amounts of cholesteryl esters accumulate so that macrophages in culture resemble foam cells found in atherosclerotic lesions^{4,5}. In an effort to identify an unmodified mammalian macromolecule that binds to the Ac LDL receptor, we investigated whether platelet secretory products affect the receptor-mediated endocytosis of chemically modified lipoproteins. Platelets are a potential source of such activity because they exist in close association with foam cells in developing atherosclerotic lesions^{6,7}. Our study demonstrates that human blood platelets secrete a product that inhibits the binding and uptake of AcAc LDL by mouse peritoneal macrophages and the subsequent accumulation of cholesteryl esters. This is the first indication that an endogenous macromolecule interacts with Ac LDL receptor on macrophages.

Platelet secretory products, obtained from platelets of freshly drawn human blood (see Fig. 1 legend), inhibited the cellular uptake of ¹²⁵I-AcAc LDL (Fig. 1a) and the subsequent degradation of the iodinated lipoprotein to trichloroacetic acid-soluble material (Fig. 1b) by mouse peritoneal macrophages. The secretory products also inhibited the uptake and degradation of ¹²⁵I-Ac LDL (data not shown). Preincubation of the macrophages with 100 μ g ml⁻¹ platelet secretory protein for 16 h followed by washing did not affect the subsequent cellular uptake and degradation of ¹²⁵I-AcAc LDL, indicating that the platelet products did not inhibit the uptake by down-regulating the Ac LDL receptor. Previous studies have shown that cultured macrophages bind AcAc LDL at 4 °C, a temperature at which internalization and subsequent proteolytic degradation do not occur⁵. We found that at 4 °C, the secretory products from platelets inhibited ¹²⁵I-AcAc LDL binding to macrophages; 59 μ g ml⁻¹ secretory products were needed for 50% inhibition (data not shown). The platelet-derived inhibitor demonstrated specificity for interaction with the Ac LDL receptor in that it had little effect on the uptake and degradation of ¹²⁵I-labelled β -migrating very-low-density lipoproteins (β -VLDL) (Fig. 1a, b, respectively) by the β -VLDL receptor on mouse peritoneal macrophages⁵. Similarly, the platelet secretory products, at concentrations shown to inhibit the uptake and degradation of ¹²⁵I-AcAc LDL by macrophages, had little effect on the uptake of ¹²⁵I-LDL by cultured human fibroblasts (data not shown). Thus, platelet secretory products act to inhibit selectively the binding, uptake and degradation of ¹²⁵I-AcAc LDL by macrophages.

The cholesterol liberated following receptor-mediated uptake of AcAc LDL by macrophages is re-esterified in the cytoplasm⁵. Cholesteryl ester synthesis can be readily assayed by including ¹⁴C-oleate in the culture medium and measuring the formation of radiolabelled cholesteryl oleate¹. We found that the platelet secretory products inhibited the AcAc LDL-induced formation of cholesteryl ¹⁴C-oleate by mouse peritoneal macrophages (Fig. 2). Therefore, one functional consequence of the platelet-derived inhibitory activity is the prevention of AcAc LDL-mediated cholesteryl ester formation and accumulation.

Agents and conditions known to affect the release of platelet secretory products were examined to determine their ability to release the inhibitor of AcAc LDL degradation (Fig. 3). Whereas the thrombin-induced secretory products inhibited the degradation of ¹²⁵I-AcAc LDL by mouse peritoneal macrophages, little

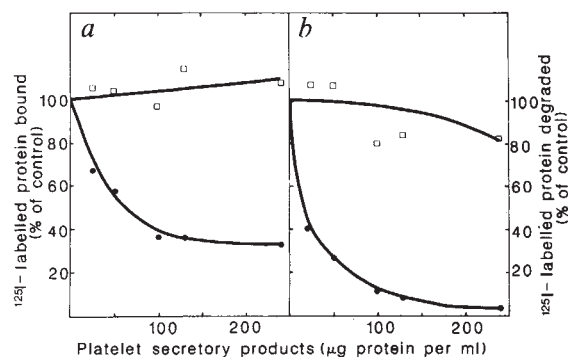


Fig. 1 Effect of platelet secretory products on the uptake (a) and degradation (b) of ^{125}I -AcAc LDL (●) or ^{125}I - β -VLDL (□) by mouse peritoneal macrophages.

Methods. Secretory products were obtained from the platelets of freshly drawn human blood. Platelets were collected and washed as described previously¹⁴ and, except where noted, suspended to a concentration of $3-5 \times 10^9$ platelets per ml in a solution containing 1 mM EDTA, 10 mM HEPES and 0.15 M NaCl pH 7.4 (EHS buffer). The washed platelets were induced to secrete by treatment with 0.5 U ml^{-1} human α -thrombin (a gift from Dr John Fenton III) for 5 min at ambient temperature, after which time the thrombin was inhibited by the addition of hirudin at 1 U ml^{-1} . The platelets were then removed by centrifugation at $10,000g$ for 15 min at 4°C and discarded. The supernatant, containing the secretory products, was dialysed against EHS buffer, concentrated by filtration (YM-10 filter, Amicon Corporation) and frozen until needed. Mouse peritoneal macrophages were collected from unstimulated mice and dispensed into 16-mm Petri dishes (5×10^5 cells per dish) as described previously¹⁵. The dishes were incubated for 2 h at 37°C and washed three times with Dulbecco's modified Eagle's medium (DMEM) to remove non-adherent cells. The cultured macrophages were incubated overnight in DMEM containing 20% fetal calf serum and washed once with DMEM before use. To measure the uptake and degradation of the labelled protein, each 16-mm dish of macrophages received 0.3 ml DMEM containing 10% human lipoprotein-deficient serum, the indicated concentrations of platelet secretory products and either $1 \mu\text{g ml}^{-1}$ ^{125}I - β -VLDL or $1 \mu\text{g ml}^{-1}$ ^{125}I -AcAc LDL. The dishes were then incubated for 5 h at 37°C . The amount of ^{125}I -labelled protein taken up and degraded by the cells was determined as described previously¹⁵. Procedures for the isolation and iodination of the AcAc LDL and β -VLDL and the modification of the LDL by acetoacetylation have also been described elsewhere¹⁵.

inhibitory activity was detected when platelets were incubated without thrombin, when thrombin was mixed with hirudin before addition to platelets, or when platelets were preincubated with prostacyclin. This demonstrates that the proteolytic activity of thrombin and the functional activity of platelets are required for the expression of the inhibitory activity. Two additional platelet agonists, the Ca^{2+} ionophore A23187 and collagen, were found to release an amount of inhibitor equivalent to that released by thrombin. These findings suggest that the inhibitor is secreted from the platelet storage organelles⁸, probably α -granules, which are the primary source of protein secreted in response to the three agonists tested⁹. Because the concentration of secretory products used for the experiment in Fig. 3 was equivalent to that induced from platelets at 2.5×10^8 per ml, we conclude that the inhibitor is secreted in sufficient concentrations at physiological platelet counts ($3-4 \times 10^8$ platelets per ml) to affect the binding activity of the Ac LDL receptor on macrophages.

Initial attempts to characterize the inhibitor of AcAc LDL uptake and degradation showed that the activity from thrombin-activated platelets persisted after samples were frozen and thawed twice. This activity was not affected by incubating the platelets for 30 min at temperatures between 4°C and 70°C and did not pass through filters (XM 100, Amicon Corporation) with an exclusion limit of relative molecular mass 100,000 (data not shown). From these observations, we tentatively conclude that the inhibitor is a stable secretory product of platelets and is

Fig. 2 Inhibition of AcAc LDL-mediated cholesterol esterification in mouse peritoneal macrophages by platelet secretory products. Each 16-mm Petri dish received 0.3 ml of DMEM containing 0.2 mM ^{14}C -oleate (oleate albumin complex), $4 \mu\text{g}$ protein per ml of AcAc LDL, and the indicated concentration of platelet secretory products. After incubation for 16 h at 37°C , the cellular content of ^{14}C -oleate was determined by TLC¹⁵.

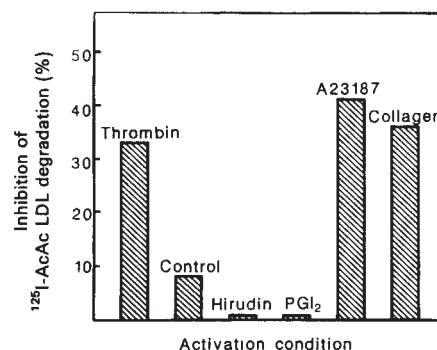
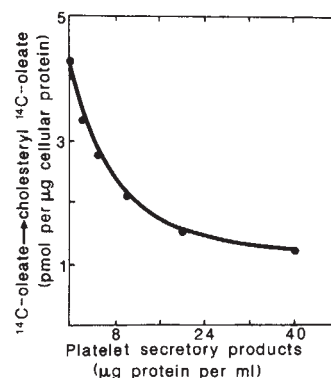


Fig. 3 Platelet secretion is required for the expression of the inhibitor of ^{125}I -AcAc LDL degradation by macrophages. Platelets from normal volunteers were isolated and suspended in EHS buffer (10^9 platelets per ml). Aliquots of 1.5 ml were treated as indicated for 5 min at ambient temperature and centrifuged at $1,000g$ for 15 min to sediment the platelets. In some cases, hirudin was added immediately after the 5-min incubation. The supernatant was removed, centrifuged at $15,000g$ for 2 min and assayed for inhibition of ^{125}I -AcAc LDL degradation as described in Fig. 1 legend, using a 1:4 dilution of the platelet supernatant. Conditions: thrombin, 0.5 U ml^{-1} ; control, no addition; thrombin plus hirudin, thrombin (0.5 U ml^{-1}) preincubated with hirudin (1 U ml^{-1}) before addition to platelets; PGI_2 , platelets preincubated with 50 nM prostacyclin for 10 min at ambient temperature before addition of thrombin; A23187, $1 \mu\text{M}$ A23187; collagen, $20 \mu\text{g ml}^{-1}$ collagen.

associated with a macromolecule(s) of high relative molecular mass.

Although the physiological function of a platelet-derived inhibitor of the Ac LDL receptor on macrophages is unknown, we suggest two possibilities. One is that this inhibitor regulates accumulation of cholesterol in these cells. In atherosclerotic lesions, cholesteryl esters accumulate in macrophage-derived foam cells^{5-7,10}. Although the mechanism of lipid uptake is unknown, it has been suggested that LDL can be modified *in vivo* so that they can be recognized by the Ac LDL receptor^{3,5,11,12}. If such modifications cause cholesterol accumulation in foam cells, the platelet secretory products released at the site of an atherosclerotic lesion could serve to dampen the rate of cholesterol accumulation. An alternative possibility is that the platelet-derived inhibitor could activate macrophages. Binding of ligands to the Ac LDL receptor on macrophages induces the secretion of several proteins, including the Ca^{2+} -dependent neutral protease, a plasminogen activator and a cytolytic factor¹³. The inhibitor from platelets may induce similar secretions.

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Cultured human endothelial cells express platelet-derived growth factor B chain: cDNA cloning and structural analysis

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Vascular endothelial cells have a central role in various pathophysiological responses such as acute inflammation, wound healing and atherogenesis. The anatomical position of endothelial cells between blood leukocytes and the surrounding vascular smooth muscle cells or stromal fibroblasts may intensify and focus the effects of released endothelial cell products. Endothelial cells in culture produce a platelet-derived growth factor (PDGF)-like mitogen¹. PDGF purified from platelets is a basic protein with an apparent relative molecular mass (M_r) of ~30,000 (reviewed in refs 2, 3) and is believed to comprise two polypeptide chains, PDGF-A and PDGF-B⁴ (also referred to as PDGF-1 and PDGF-2; refs 5, 6). Sequence analysis of PDGF B chain has revealed a striking homology with the predicted sequence of p28^{sis}, the transforming protein of simian sarcoma virus^{4,6,7}. *sis*-Homologous transcripts have been detected by Northern blot analysis of RNA from cultured endothelial cells⁸. However, there are no structural data available on either the protein product or the messenger RNA to establish the identity of the endothelial-derived mitogen with either chain of PDGF. Here we report the isolation and complete sequence analysis of a *sis*-homologous complementary DNA clone from human endothelial cells, providing an opportunity to study the structure of *sis* as transcribed by a normal (untransformed) cell. Our results establish that normal human endothelial cells in culture express the B chain of PDGF, and that endothelial-derived PDGF B chain is synthesized as a predicted precursor polypeptide of M_r 27,281.

A prominent *v-sis* hybridizing band of about 3.5 kilobases (kb) was detected in Northern blots of cytoplasmic RNA from cultured human umbilical vein endothelial (HUVE) cells⁹, a simian virus 40 (SV40)-transformed endothelial cell line¹⁰ and a bovine aortic⁹ endothelial cell strain (Fig. 1A). These bands are of a similar size to that of an RNA species isolated from a human osteosarcoma cell line (HOS), previously reported to be ~4.2 kb¹¹. A diffuse minor band of 2.7 kb was also observed. No *v-sis* hybridizing bands were detected in RNA preparations from cultured human dermal fibroblasts or from a B-lymphoblastoid cell line.

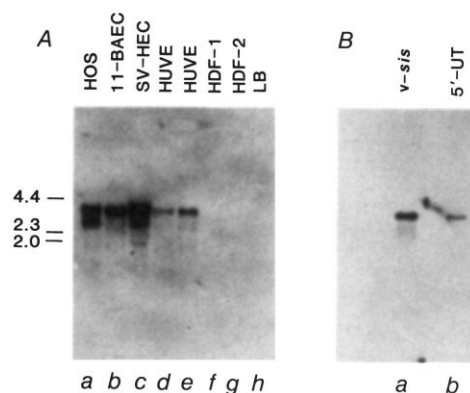


Fig. 1 A, Expression of *c-sis* in cultured vascular endothelial cells. *a-c*, 50 µg each of cytoplasmic RNA from a human osteosarcoma cell line (HOS), cultured bovine aortic endothelial cells (BAEC) and an SV40-transformed endothelial cell line, respectively; *d, e*, 10 and 20 µg, respectively, of cytoplasmic RNA from human umbilical vein endothelial cells; *f, g*, 50 µg of cytoplasmic RNA from each of two strains of human dermal fibroblasts; *h*, 5 µg of poly(A)⁺ RNA from the lymphoblastoid line LB. The molecular sizes (kb) of ³²P 3'-end-labelled *Hind*III-digested phage λ DNA, processed in parallel with the RNA samples, are indicated on the left. B, Hybridization probes from the 5'-untranslated region of the B2-1 clone and the B-chain coding region identify the same transcript in RNA from HUVE cells. In each lane, 5 µg of poly(A)⁺ RNA from HUVE cells was analysed by RNA blotting techniques³⁰. Following transfer to nitrocellulose, the lanes were separated and independently analysed with: *a*, a coding-region probe (*v-sis*) or *b*, a probe from the 5'-untranslated (5'-UT) region (an *Eco*RI-*Hin*I restriction fragment, nucleotides 1-280).

Methods. Total cytoplasmic or poly(A)⁺ RNA was prepared as described elsewhere³⁰. The RNAs were denatured with formaldehyde and electrophoresed in a 1% agarose gel, transferred to nitrocellulose, and probed with nick-translated cDNA inserts as described elsewhere³⁰. The filter was washed in 0.5 × SSC at 68 °C and exposed to film as described previously³¹.

To determine the nucleotide sequence of the *sis*-homologous 3.5-kb transcript, a cDNA corresponding to the endothelial cell mRNA was cloned and its primary structure determined. Restriction mapping and Southern blot hybridization with the *v-sis* probe were used to establish the overlapping orientation of two cDNA clones from the random hexanucleotide (B2-1) and the oligo(dT)-primed (A1-6) libraries (Fig. 2). The 5' clone (B2-1) contains the *v-sis*-homologous region and extends 860 base pairs (bp) farther 5' than a previously described cDNA clone derived from the HUT 102 neoplastic T-cell line¹². The 3' clone A1-6 ends just 3' to an *Sst*I site located ~500 bp upstream from the polyadenylation site (based on a restriction map comparison with the HUT 102 cDNA). Together these clones span 3.2 kb and by inference account for most of the 3.5-kb *sis*-homologous transcript observed on Northern blots of HUVE cytoplasmic RNA.

Sequence analysis of the B2-1 clone confirms the presence of a region of *v-sis* homology containing an open reading frame that encodes the B chain of PDGF; it also reveals an exceptionally long 5'-untranslated region (Fig. 3A). The *v-sis*-homologous region begins at position 1,053 and continues to the 3' end of this clone. Over the region encoding the B-chain protein sequence, the HUVE cDNA clone is identical in nucleotide sequence both to the previously sequenced clone from the HUT 102 T lymphoma cell line¹² and to the exons of the normal human *c-sis* gene^{4,13,14}. A potential initiation codon, ATG (position 989), is in-phase with the B-chain open reading frame and the coding region would extend 723 bp before terminating at position 1,712. The predicted primary translation product for the B chain is 241 amino acids long (M_r 27,281). The sequence in Fig. 2 must encode the entire precursor for the B chain because, as in HUT 102, an in-frame translational stop codon is located 117 bp upstream (position 872) from the open reading frame.

The 5'-untranslated region of the PDGF B-chain transcript is unusually long (at least 983 bp). Most 5'-untranslated regions are less than ~150 bp long¹⁸, although there are notable exceptions. Specifically, *c-myc*, another cellular homologue of a retroviral transforming gene (whose expression is activated by PDGF¹⁹), has a 550-bp 5'-untranslated region²⁰. Another mitogen, murine preproepidermal growth factor, also has a long (353 bp) 5'-untranslated region²¹, and genomic sequence analysis suggests that the insulin-like growth factor II precursor has ~1 kb of 5'-untranslated region²². The transcript is also unusual in that translation begins at the fourth most 5'-proximal ATG codon (position 989, Fig. 3B). Recently, a consensus sequence (CC(A or G)CCATGG) for translation initiation has been defined¹⁸, in which purine nucleotides three positions upstream and one position downstream from the ATG are particularly important²³. Only the ATG in the B-chain reading frame meets both these criteria (TCGGCATGA). The second open reading frame could encode a protein of *M_r* 12,529. A search of the GenBank sequence database revealed no significantly (non-sis-related) homologous sequences. In addition, analysis of codon usage²⁴ suggests that this open reading frame is unlikely to encode a translated protein.

The extreme length of the 5'-untranslated region and the presence of three other ATG codons may be important in the physiological regulation of PDGF expression. Interestingly, both the naturally transforming retrovirus^{15,25} and the experimentally transforming HUT 102 cDNA²⁶ clone delete the other upstream ATG codons. Similarly, when *c-sis* genomic constructions lacking the complete 5'-untranslated region are placed under the transcriptional control of a retroviral long terminal repeat, the constructs acquire high-titre transforming activity¹⁶.

PDGF as purified from human platelets contains a second polypeptide that is less homologous to *v-sis* (A chain)⁴, and it is thought that human PDGF is predominantly a 1:1 disulphide-linked heterodimer of A and B chains^{2,3}. The cDNA sequence reported here establishes that the A chain does not arise from the same precursor polypeptide as the B chain of PDGF. The data presented here do not, however, exclude the possibility that *c-sis* encodes the A chain of PDGF, as it could be generated by differential splicing. It is unknown whether endothelial cell-derived PDGF-like mitogen is also an A/B chain mixture, or whether endothelial cells even synthesize the A chain of PDGF. It is possible that the endothelial mitogen is a B-chain homodimer, as has been suggested for porcine PDGF²⁷. The eventual generation of complete PDGF A-chain and B-chain cDNA clones may elucidate both the functional interactions between these polypeptides and the potential issue of coordinate expression. Finally, the 5' cDNA sequence information presented here should assist in locating the true 5' end of the human *c-sis* gene and, with it, possible sequences that regulate endothelial cell PDGF expression *in situ*^{8,28}. Such information may be critical in understanding the role of endothelium in pathological processes.

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Light-regulated and organ-specific expression of a wheat *Cab* gene in transgenic tobacco

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Many of our most important crop plants are monocotyledons, including wheat, corn, rice and barley. No routine transformation system for monocotyledons has been reported, such as the Ti-mediated gene transfer system for dicotyledons facilitated by *Agrobacterium tumefaciens*¹⁻³. Indirect evidence suggests that Ti-plasmid DNA is transferred into and expressed in *A. tumefaciens*-infected wound tissues of plants from Liliaceae and Amaryllidaceae^{4,5}, but these observations have not been extended to monocotyledons of greatest agricultural importance. Regeneration of monocotyledons is usually blocked at the callus stage, further complicating the possibility of exploring the regulated expression of their genes, and thus preventing identification of the regulatory domains of monocotyledonous genes in a homologous nuclear background. To circumvent these difficulties, we investigated whether monocotyledonous genes can be expressed and correctly regulated in dicotyledons. We have introduced a wheat gene (*whAB1.6*)⁶ encoding the major chlorophyll *a/b* binding protein (*Cab*) of the light-harvesting complex into the genomes of tobacco (*Nicotiana tabacum* SR1) and petunia (*Petunia hybrida*) via a Ti-DNA-mediated gene transfer system which allows the transformed cells to regenerate into whole plants⁷. Here we report for the first time the light-regulated and organ-specific expression of a monocotyledonous gene in transgenic dicotyledonous plants.

A 6.5-kilobase (kb) wheat genomic fragment containing the *Cab* gene (*whAB1.6*) was ligated into the *Cl*A site of the vector pMON145 (Fig. 1)⁸ and the construct was mobilized into a 'disarmed' *A. tumefaciens* derivative (GV311ISE)⁹. After co-cultivation of *A. tumefaciens* and protoplasts of *N. tabacum* SR1, transformed cells were identified by their resistance to kanamycin at 100 µg ml⁻¹ (ref. 1). Plants were regenerated from transformed calli according to Marton *et al.*⁹.

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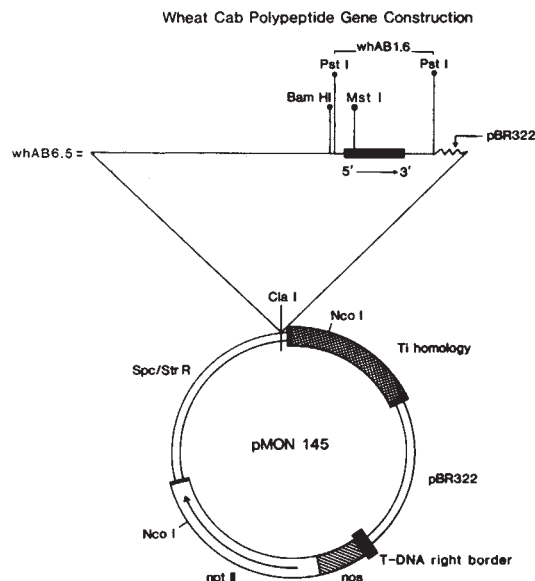


Fig. 1 Wheat Cab gene construction used for transformation. An 8.7-kb wheat genomic fragment containing the Cab gene, *whAB1.6* (ref. 6), was subcloned into pBR322 to yield the construct *whAB8.7*. Digestion of *whAB8.7* with *Cla*I releases a 6.5-kb fragment containing the entire wheat Cab protein-coding sequence, 4.4 kb upstream of the transcription start site, 800 bp beyond the stop codon and 250 bp of pBR322 at the 3' end. The 6.5-kb *Cla*I fragment was inserted into the *Cla*I site of pMON145, which has been described in detail elsewhere⁸. pMON145 contains the *NOS-NptII* fusion gene, a region homologous to *A. tumefaciens* Ti-DNA, a region of pBR322, and spectinomycin (*spc*) and streptomycin (*str*) resistance genes. It also contains a 400-bp segment (*Pvu*I/*Sst*II) of the pTiT37 *Hind*III-23 fragment which includes the pTiT37 T-DNA right border⁸.

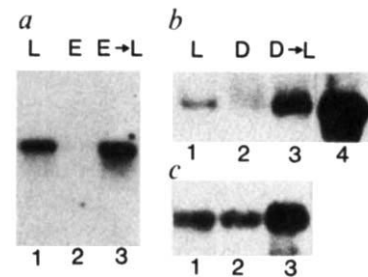
Methods. *Escherichia coli* strain LE392 was transformed with the *whAB6.5/pMON145* plasmid construct, and colonies were selected for *spc* and *str* resistance. The *whAB6.5/pMON145* construct was introduced by a triparental cross⁸ into a disarmed *A. tumefaciens* derivative (GV3111SE) containing a modified Ti-plasmid lacking phytohormone biosynthetic genes and TL-DNA right border⁷.

Northern blot analysis shows that in wheat the accumulation of Cab transcripts is strongly dependent on light (Fig. 2a). Using as a probe a labelled *Bgl*II/*Sma*I fragment, which encodes the carboxy-terminus of the mature Cab protein⁶, the amount of Cab transcripts in etiolated leaves is at least 20 times less than in green leaves. When etiolated wheat plants are transferred to the light for 24 h, leaf Cab messenger RNA increases to the amount that accumulates in leaves from plants grown in a normal light/dark cycle. The Northern blot analysis does not discriminate among transcripts from different members (at least seven) of the wheat Cab multigene family⁶.

To establish whether expression of *whAB1.6* is light-dependent, a gene-specific *Bam*HI/*Mst*I fragment was hybridized to the same RNAs and the products analysed by the *S*₁ nuclease assay¹⁰. The *Bam*HI/*Mst*I probe is 305 nucleotides long, and yields an *S*₁-resistant product of 169 nucleotides which includes the *whAB1.6* non-translated leader and extends 102 nucleotides into the Cab coding region. Figure 3a shows that this product is found only after light stimulation. We estimate that the *whAB1.6* transcript level increases at least 20-fold in going from the dark to the light.

To determine whether the wheat Cab gene is expressed in leaves of transgenic tobacco, and also whether it is light-regulated, we isolated RNA from leaves pooled from 10 individual transgenic tobacco clones. The plants were (1) grown under a light/dark, 16/8-h photoperiodic cycle, (2) transferred to the dark for 7 days and (3) transferred to the dark and then re-exposed to continuous light for 24 h. Blots of these RNAs were hybridized with the labelled *Bgl*II/*Sma*I fragment from *whAB1.6* (ref. 6), which does not cross-hybridize with the endogenous tobacco Cab mRNA in stringent conditions (Fig.

Fig. 2 Northern blot analysis of transcripts from wheat and transgenic tobacco leaves. *a*, Total RNA (10 µg) from wheat leaves of plants grown in the light (L) for 8 days in a 16/8-h light/dark cycle (lane 1), etiolated (E) plants grown in the dark (lane 2), or etiolated plants transferred to the light (E→L) for 24 h (lane 3). *b*, RNA (10 µg) from leaves of transgenic tobacco plants grown in a 16/8-h light/dark cycle from the callus stage (lane 1), young plants grown in the dark for 7 days (lane 2) and young plants transferred from the dark to the light (D→L) for 24 h (lane 3). Lane 4 shows a wheat leaf RNA (10 µg) control included in the tobacco analysis. *c*, Poly(A) RNAs were prepared¹¹ from leaves of transgenic tobacco plants. Lanes 1–3 are the same as lanes 1–3 in *b*. Each lane contained 1 µg of poly(A) RNA.



Methods. Total RNA was isolated from either wheat or tobacco leaves using the guanidinium thiocyanate procedure¹⁹, and Northern blots were analysed according to Carmichael and McMaster²⁰. The blots for *a* and *b* were hybridized to a ³²P-labelled *Bgl*II/*Sma*I fragment (10⁸ c.p.m. per µg) of 383 bp and encoding the carboxy-terminus of the Cab protein⁶. Hybridization was performed in 50% formamide, 2×Denhardt's, 50 mM NaPO₄, and 5×SSC, 100 µg ml⁻¹ calf thymus DNA at 46 °C. In these conditions the fragment does not hybridize with tobacco RNA (see Fig. 4a, lane 7). The blot for *c* was hybridized to a nick-translated probe containing the coding region of *NptII* in the same conditions.

4a, lane 7). Figure 2b demonstrates that the wheat Cab gene is expressed in light-grown tobacco leaves at a level at least 4–10-fold higher than in leaves of dark-adapted plants (compare Fig. 2b, lanes 1, 2, and Fig. 4b, lanes 1, 2). On re-illumination, the wheat Cab mRNA level is often more than double the level found in plants grown in a 16/8-h light/dark photoperiodic cycle. The reason for this mRNA overproduction is being investigated. The wheat Cab mRNA level is reduced 4-fold in tobacco plants transferred to the dark and then increases 10-fold when plants are re-illuminated (Fig. 2b). To determine whether the reduction of the wheat Cab transcript level in the dark is selective, we prepared poly(A) RNAs¹¹ and analysed the mRNA levels for the chimaeric gene comprised of the nopaline synthase (*NOS*) promoter fused to the neomycin phosphotransferase (*NptII*) coding sequence (*NOS-NptII*). Figure 2c shows that the *NOS-NptII* transcript is indeed present in the dark-adapted plants and in plants returned to the light. The *NOS-NptII* gene has been demonstrated to be constitutively expressed in all tissues of plants which are kanamycin-resistant¹², including those transferred to the dark (F.N., unpublished results).

The Northern blot analyses demonstrate the presence of the wheat Cab transcript in transgenic tobacco leaves and that it is the same size (1,100 nucleotides) as in wheat. Furthermore, the wheat Cab mRNA can be recovered in the tobacco poly(A) RNA fraction (data not shown). These results suggest that the wheat Cab transcript is correctly processed and polyadenylated. To ascertain if accurate transcription initiation also occurs, transcripts from transgenic tobacco were mapped by *S*₁ nuclease protection. A 5' end-labelled *whAB1.6 Bam*HI/*Mst*I fragment, described above, was hybridized with total leaf RNA from both light- and dark-grown plants, and the products were analysed alongside those obtained using wheat leaf RNA. A 169-nucleotide *S*₁-resistant product is obtained with either the wheat or the transgenic tobacco leaf RNAs (Fig. 3b). We conclude that transcription of the wheat Cab gene begins correctly in tobacco, and confirm with the *S*₁ nuclease results that the level of the wheat Cab transcript is also controlled by light in the transgenic plants. The transcripts are present in transgenic tobacco at ~10–25% the level found in wheat leaves (Fig. 3, compare lanes 1, 3, 5). These values do not reflect the absolute expression level of the wheat Cab gene because its copy numbers in the transgenic tobacco plants have not been determined. In contrast, the *NOS*-

transcript is indeed present in the dark-adapted plants and in plants returned to the light. The *NOS-NptII* gene has been demonstrated to be constitutively expressed in all tissues of plants which are kanamycin-resistant¹², including those transferred to the dark (F.N., unpublished results).

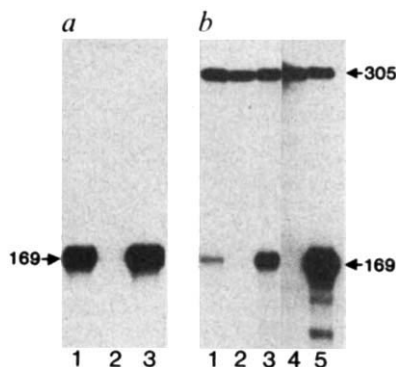


Fig. 3 S_1 nuclease analysis of light-regulated wheat Cab transcripts in wheat and transgenic tobacco. *a*, S_1 products using wheat RNAs. The same RNAs (10 μ g per lane) for lanes 1, 2 and 3 were used as described in Fig. 2a legend. *b*, S_1 products using transgenic tobacco RNAs. Lanes 1–3 are analogous to the lanes in Fig. 2b. Lane 4 shows hybridization of the *Bam*HI/*Mst*I fragment to untransformed tobacco RNA, and lane 5, a wheat leaf RNA control reaction. Each lane contained 10 μ g RNA. The band of high relative molecular mass (M_r) (305 nucleotides) corresponds to the undigested probe. The S_1 nuclease-resistant band at 169 nucleotides is caused by hybridization of the probe to the *whAB1.6*-specific transcript and the low- M_r bands (lane 5) represent hybrids between the probe and transcripts of other wheat Cab genes that show limited sequence homology to *whAB1.6* in the non-translated leader RNA region⁶.

Methods. RNAs were prepared from wheat and tobacco as described for Fig. 2. A 5' end-labelled *Bam*HI/*Mst*I fragment from *whAB1.6* (ref. 6) was hybridized with 25 μ g of total RNA in 40 mM PIPES (pH 6.4), 1 mM EDTA, 0.4 M NaCl, 80% formamide. Samples were incubated for 4 h at 50°C, digested with 30 units of S_1 nuclease (BRL) at 37°C, in 0.3 M NaCl, 4.5 mM ZnCl₂, 5 mM sodium acetate and 20 μ g ml⁻¹ salmon sperm DNA¹⁰, and extracted with phenol/chloroform and ether. S_1 nuclease-resistant products were precipitated with 25 μ g of *E. coli* transfer RNA and aliquots were analysed on 8% acrylamide gels.

NptII transcripts can be detected only in the poly(A) RNA fraction (Fig. 2), which requires a ~100-fold enrichment step.

whAB1.6 is active in wheat leaves, requires light for activation (Fig. 3a) and is not expressed in roots⁶. To establish if the wheat Cab gene construct also contains sufficient information for organ-specific expression in the transgenic plants, RNAs were isolated from different organs of transgenic tobacco. We found that the wheat Cab gene is expressed predominantly in tobacco leaves by Northern blot analyses (Fig. 4a) and by S_1 nuclease protection assays (data not shown). In some RNA preparations the wheat Cab mRNA can be detected in tobacco stems at a lower level (~5–10-fold) than in leaves; expression continues to be light-regulated (Fig. 4b). We have also transferred the wheat Cab gene into *P. hybrida* and obtained accurate transcriptional initiation and light-regulated and organ-specific expression (data not shown).

Our results demonstrate that a wheat Cab gene is accurately transcribed and selectively expressed in transgenic tobacco and petunia. This is the first report of regulated expression of any Cab gene in transgenic plants. Our observations indicate that the dicotyledonous nuclear transcription apparatus recognizes the relevant regulatory sequences of the wheat Cab gene, and that the necessary information for gene activation is contained within a wheat genomic fragment which begins 4.4 kb 5' to the transcription initiation site and extends 850 base pairs (bp) beyond the Cab protein translation stop codon. Furthermore, the wheat Cab transcripts are polyadenylated (data not shown) and stable in tobacco and petunia cells.

A zein gene from maize is expressed in sunflower calli¹³, although it is not known whether organ-specificity of the zein gene will be retained in regenerated plants. Our finding that the wheat Cab gene functions with specificity in two dicotyledonous plants suggests that at least some dicotyledonous genes may show regulated expression in monocotyledons.

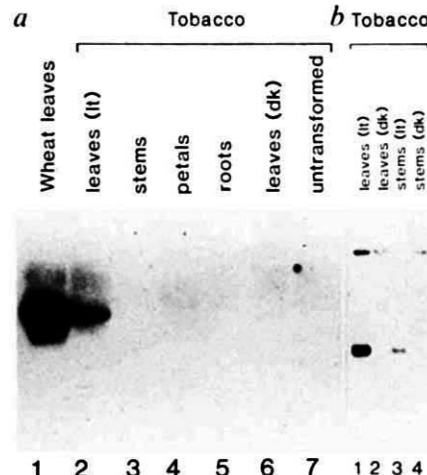


Fig. 4 Organ-specific expression of the wheat Cab gene in tobacco. *a*, Northern blot analysis. Lane 1, wheat leaves; lanes 2–5, leaves, stems, petals and roots, respectively, from transgenic tobacco plants grown under a 16/8-h light/dark photoperiod; lane 6, leaves of transgenic tobacco plants transferred to the dark for 7 days; lane 7, leaves of untransformed tobacco plants. Each lane contained 10 μ g RNA. *b*, S_1 nuclease protection assays. Lanes 1, 3, leaves and stems of transgenic tobacco grown under a 16/18-h light/dark photoperiod; lanes 2, 4, leaves and stems of transgenic tobacco transferred to the dark for 7 days. Each lane contained 10 μ g RNA.

Methods. RNAs were extracted from different plant organs¹⁹ and analysed by Northern blot hybridizations²⁰ or S_1 nuclease protection assays¹⁰. See Figs 2 and 3 legends for details.

The *cis*-acting sequences that control the wheat Cab gene have not been characterized. That the regulatory sequences of the wheat Cab gene functions with apparent fidelity in tobacco and petunia renders their identification feasible by combining *in vitro* mutagenesis and the *A. tumefaciens*-mediated gene transfer system, as has been done for the genes encoding the ribulose 1,5-bisphosphate carboxylase small subunit of pea (*rbcS*)^{14,21} and the 35S promoter of cauliflower mosaic virus¹². Our immediate goal is to compare the DNA sequences used for the light-dependent expression of the Cab¹⁵ and *rbcS* (refs 16, 17) genes. In peas the accumulation of Cab and *rbcS* transcripts begins at different red-light fluencies¹⁸, which suggests that the genes encoding key components of the chloroplast photosynthetic machinery are selectively responsive to light and regulated independently during shoot development.

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